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(71) Applicant: VIAGENE, INC. [US/US]; 11075 Roselle Street, San Diego, CA 92121 (US).

(72) Inventors: GRUBER, Harry, E. ; 13083 Maritime Place, San Diego, CA 92130 (US). JOLLY, Douglas, J. ; 3050HDJJ Via Alicante Drive, La Jolla, CA 92037 (US). RESPESS, James, G. ; 4966 Lamont Street, San Diego, CA 92109 (US). LAIKIND, Paul, K. ; 12433 Caminito Mira Del Mar, San Diego, CA 92130 (US). CHANG, Stephen, M., W. ; 9838 Via Caceras, San Diego, CA 92129 (US). CHADA, Sunil, D. ; 1542 Enchantment Avenue, Vista, CA 92083 (US). WARNER, John, F. ; 4125 Pilon Point, San Diego, CA 92130 (US). BARBER, Jack, R. ; 11168 Carlotta Street, San Diego, CA 92129 (US). ST. LOUIS, Daniel, C. ; 1500 Gude Drive, Rockville, MD 20850 (US).

(74) Agents: MAKI, David, J. et al.; Seed and Berry, 6300 Columbia Center, Seattle, WA 98104-7092 (US).

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(54) Title: RECOMBINANT RETROVIRUSES DELIVERING VECTOR CONSTRUCTS TO TARGET CELLS

(57) Abstract

Recombinant retroviruses carrying a vector construct capable of preventing, inhibiting, stabilizing or reversing infectious, cancerous or auto-immune diseases are disclosed. More specifically, the recombinant retroviruses of the present invention are useful for (a) stimulating a specific immune response to an antigen or a pathogenic antigen; (b) inhibiting a function of a pathogenic agent, such as a virus; and (c) inhibiting the interaction of an agent with a host cell receptor. In addition, eucaryotic cells infected with, and pharmaceutical compositions containing such a recombinant retrovirus are disclosed. Various methods for producing recombinant retroviruses having unique characteristics, and methods for producing transgenic packaging animals or insects are also disclosed.

RETROVIRAL CONSTRUCTS OF ENV^R

DESIGNATIONS OF "DE"

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Description

RECOMBINANT RETROVIRUSES DELIVERING VECTOR CONSTRUCTS TO TARGET CELLS

Technical Field

The present invention relates generally to retroviruses, and more specifically, to recombinant retroviruses which are capable of delivering vector constructs to susceptible target cells. These vector constructs are typically designed to express desired proteins in target cells, for example, proteins which stimulate immune activity or which are conditionally active in defined cellular environments. In these respects the retrovirus carrying the vector construct is capable of directing an immune or toxic reaction against the target cell.

Background of the Invention

Although bacterial diseases are, in general, easily treatable with antibiotics, very few effective treatments or prophylactic measures exist for many viral, cancerous, and other nonbacterial diseases, including genetic diseases. Traditional attempts to treat these diseases have employed the use of chemical drugs. In general, these drugs have lacked specificity, exhibited high overall toxicity, and thus have been therapeutically ineffective.

Another classic technique for treating a number of nonbacterial diseases involves the elicitation of an immune response to a pathogenic agent, such as a virus, through the administration of a noninfectious form of the agent, such as a killed virus, thereby providing antigens from the pathogenic agent which would act as an immuno-stimulant.

A more recent approach for treating viral diseases, such as acquired immunodeficiency syndrome

(AIDS) and related disorders, involves blocking receptors on cells susceptible to infection by HIV from receiving or forming a complex with viral envelope proteins. For example, Lifson et al. (Science 232:1123-1127, 1986)

5   demonstrated that antibodies to CD4 (T4) receptors inhibited cell fusion (syncytia) between infected and noninfected CD4 presenting cells in vitro. A similar CD4 blocking effect using monoclonal antibodies has been suggested by McDougal et al. (Science 231:382-385, 1986).

10 Alternatively, Pert et al. (Proc. Natl. Acad. Sci. USA 83:9254-9258, 1986) have reported the use of synthetic peptides to bind T4 receptors and block HIV infection of human T-cells, while Lifson et al. (J. Exp. Med. 164:2101, 1986) have reported blocking both syncytia and virus/T4

15 cell fusion by using a lectin which interacts with a viral envelope glycoprotein, thereby blocking it from being received by CD4 receptors.

A fourth, recently suggested technique for inhibiting a pathogenic agent, such as a virus, which

20 transcribes RNA is to provide antisense RNA which complements at least a portion of the transcribed RNA, and binds thereto, so as to inhibit translation (To et al., Mol. Cell. Biol. 6:758, 1986).

However, a major shortcoming of the techniques

25 described above is that they do not readily lend themselves to control as to the time, location or extent to which the drug, antigen, blocking agent or antisense RNA are utilized. In particular, since the above techniques require exogenous application of the treatment agent

30 (i.e., exogenous to the sample in an in vitro situation), they are not directly responsive to the presence of the pathogenic agent. For example, it may be desirable to have an immunostimulant expressed in increased amounts immediately following infection by the pathogenic agent.

35 In addition, in the case of antisense RNA, large amounts would be required for useful therapy in an animal, which under current techniques would be administered without

regard to the location at which it is actually needed,
that is, at the cells infected by the pathogenic agent.

As an alternative to exogenous application,
techniques have been suggested for producing treatment
5   agents endogenously.   More specifically, proteins
expressed from viral vectors based on DNA viruses, such as
adenovirus, simian virus 40, bovine papilloma, and
vaccinia viruses, have been investigated.   By way of
example, Panicali et al. (Proc. Natl. Acad. Sci. USA
10 80:5364, 1983) introduced influenza virus hemagglutinin
and hepatitis B surface antigens into the vaccinia genome
and infected animals with the virus particles produced
from such recombinant genes.   Following infection, the
animals acquired immunity to both the vaccinia virus and
15 the hepatitis B antigen.

However, a number of difficulties have been
experienced to date with viral vectors based on DNA
viruses.   These difficulties include (a) the production of
other viral proteins which may lead to pathogenesis or the
20 suppression of the desired protein; (b) the capacity of
the vector to uncontrollably replicate in the host, and
the pathogenic effect of such uncontrolled replication;
(c) the presence of wild-type virus which may lead to
viremia; and (d) the transitory nature of expression in
25 these systems.   These difficulties have virtually
precluded the use of viral vectors based on DNA viruses in
the treatment of viral, cancerous, and other nonbacterial
diseases, including genetic diseases.

Due to the nontransitory nature of their
30 expression in infected target cells, retroviruses have
been suggested as a useful vehicle for the treatment of
genetic diseases (for example, see F. Ledley, The Journal
of Pediatrics 110:1, 1987).   However, in view of a number
of problems, the use of retroviruses in the treatment of
35 genetic diseases has not been attempted.   Such problems
relate to (a) the apparent need to infect a large number
of cells in inaccessible tissues (e.g., brain); (b) the

need to cause these vectors to express in a very controlled and permanent fashion; (c) the lack of cloned genes; (d) the irreversible damage to tissue and organs due to metabolic abnormalities; and (e) the availability
5   of other partially effective therapies in certain instances.

In addition to genetic diseases, other researchers have contemplated using retroviral vectors to treat nongenetic diseases (see, for example, EP 243,204 –
10 Cetus Corporation; Sanford, J. Theor. Biol. 130:469, 1988; Tellier et al., Nature 318:414, 1985; and Bolognesi et al., Cancer Res. 45:4700, 1985).

Tellier et al. suggested protecting T-cell clones by apparently infecting stem cells with "defective"
15 HIV having a genome which could express antisense RNA to HIV RNA. Bolognesi et al. have suggested the concept of generating a nonvirulent HIV strain to infect stem cells so that T4 cells generated therefrom would carry interfering, nonvirulent forms of virus and thereby
20 protect those cells from infection by virulent HIV. However, it would appear that the "attenuated" or "defective" HIV viruses used in both of the foregoing papers could reproduce (i.e., are not replication defective) such that the resulting viruses could infect
25 other cells, with the possibility of an increased risk of recombination with previously present HIV or other sequences, leading to loss of attenuation. Non-nonreplicative forms would necessitate a defective helper or packaging line for HIV. However, since the control of
30 HIV gene expression is complex, such cells have to date not been constructed. Furthermore, as the infecting attenuated or defective virus is not chimeric (a "nonchimeric" retrovirus being one with substantially all of its vector from the same retrovirus species), even if
35 they were made replication defective, for example, by deletion from their genomes of an essential element, there still exists a significant possibility for recombination

within the host cells with resultant production of infectious viral particles.

Although Sanford (<u>J. Theor. Biol</u>. <u>130</u>:469, 1988) has also proposed using a genetic cure for HIV, he notes that due to the potential that exists for creating novel virulent viruses via genetic recombination between natural AIDS virus and therapeutic retroviral vectors carrying anti-HIV genes, retroviral gene therapy for AIDS may not be practical. Similarly, while McCormick & Kriegler (EP 243,204 A2) have proposed using retroviral vectors to deliver genes for proteins, such as tumor necrosis factor (TNF), the techniques they describe suffer from a number of disadvantages.

Summary of the Invention

Briefly stated, the present invention provides recombinant retroviruses carrying a vector construct capable of preventing, inhibiting, stabilizing or reversing infectious, cancerous, auto-immune or immune diseases. Such diseases include HIV infection, melanoma, diabetes, graft vs. host disease, Alzheimer's disease, and heart disease.

The present invention is directed, in part, toward methods for (a) stimulating a specific immune response, either humoral or cell-mediated, to an antigen or pathogenic antigen; (b) inhibiting a function of a pathogenic agent, such as a virus; and (c) inhibiting the interaction of an agent with a host cell receptor, through the use of recombinant retroviruses.

More specifically, within one aspect of the present invention, a method for stimulating a specific immune response is provided, comprising infecting suscep-tible target cells with recombinant retroviruses carrying a vector construct that directs the expression of an antigen or modified form thereof in infected target cells. For purposes of the present invention, the term "infecting" includes the introduction of nucleic acid

sequences through viral vectors, transfection or other means, such as microinjection, protoplast fusion, etc. The introduced nucleic acid sequences may become integrated into the nucleic acid of the target cell.

5 Expression of the vector nucleic acid encoded protein may be transient or stable with time. Where an immune response is to be stimulated to a pathogenic antigen, the recombinant retrovirus is preferably designed to express a modified form of the antigen which will stimulate an
10 immune response and which has reduced pathogenicity relative to the native antigen. This immune response is achieved when cells present antigens in the correct manner, i.e., in the context of the MHC class I and/or II molecules along with accessory molecules such as CD3,
15 ICAM-1, ICAM-2, LFA-1, or analogs thereof (e.g., Altmann et al., Nature 338:512, 1989). Cells infected with retroviral vectors are expected to do this efficiently because they closely mimic genuine viral infection.

This aspect of the invention has a further
20 advantage over other systems that might be expected to function in a similar manner, in that the presenter cells are fully viable and healthy, and no other viral antigens (which may well be immunodominant) are expressed. This presents a distinct advantage since the antigenic epitopes
25 expressed can be altered by selective cloning of sub-fragments of the gene for the antigen into the recombinant retrovirus, leading to responses against immunogenic epitopes which may otherwise be overshadowed by immunodominant epitopes. Such an approach may be extended
30 to the expression of a peptide having multiple epitopes, one or more of the epitopes derived from different proteins. Further, this aspect of the invention allows efficient stimulation of cytotoxic T lymphocytes (CTL) directed against antigenic epitopes, and peptide fragments
35 of antigens encoded by sub-fragments of genes, through intracellular synthesis and association of these peptide fragments with MHC Class I molecules. This approach may

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be utilized to map major immunodominant epitopes for CTL. In addition, the present invention provides for a more efficient presentation of antigens through the augmentation or modification of the expression of
5   presenting accessory proteins (e.g., MHC I, ICAM-1, etc.) in antigen presenting cells. Such an approach may involve a recombinant retrovirus carrying a vector construct which directs expression of both an antigen (e.g., a tumor antigen) and an MHC protein (e.g., Class I or II) capable
10 of presenting the antigen (or a portion thereof) effectively to T lymphocytes so that it stimulates an immune response in an animal. This offers the advantage that antigen presentation may be augmented in cells (e.g., tumor cells) which have reduced levels of MHC proteins and
15 a reduced ability to stimulate an immune response. The approach may additionally involve a recombinant retrovirus carrying a vector construct which directs expression of both an antigen and a protein stimulating increased MHC protein expression in cells (e.g., interferon). The
20 retrovirus infected cells may be used as an immunostimulant, immunomodulator, or vaccine, etc.

An immune response can also be achieved by transferring to an appropriate immune cell (such as a T lymphocyte) the gene for the specific T-cell receptor
25 which recognizes the antigen of interest (in the context of an appropriate MHC molecule if necessary), for an immunoglobulin which recognizes the antigen of interest, or for a hybrid of the two which provides a CTL response in the absence of the MHC context.
30   In the particular cases of disease caused by HIV infection, where immunostimulation is desired, the antigen generated from the recombinant retroviral genome is of a form which will elicit either or both an HLA class I- or class II-restricted immune response. In the case of HIV
35 envelope antigen, for example, the antigen is preferably selected from gp 160, gp 120, and gp 41, which have been modified to reduce their pathogenicity. In particular,

the antigen selected is modified to reduce the possibility of syncytia, to avoid expression of epitopes leading to a disease enhancing immune response, to remove immunodominant, but strain-specific epitopes or to present
5 several strain-specific epitopes, and allow a response capable of eliminating cells infected with most or all strains of HIV. The strain-specific epitopes can be further selected to promote the stimulation of an immune response within an animal which is cross-reactive against
10 other strains of HIV. Antigens from other HIV genes or combinations of genes, such as gag, pol, rev, vif, nef, prot, gag pol, gag prot, etc., may also provide protection in particular cases.

In another aspect of the present invention,
15 methods for inhibiting a function of a pathogenic agent necessary for disease, such as diseases caused by viral infections, cancers or immunological abnormalities, are disclosed. Where the pathogenic agent is a virus, the inhibited function may be selected from the group
20 consisting of adsorption, replication, gene expression, assembly, and exit of the virus from infected cells. Where the pathogenic agent is a cancerous cell or cancer-promoting growth factor, the inhibited function may be selected from the group consisting of viability, cell
25 replication, altered susceptibility to external signals, and lack of production of anti-oncogenes or production of mutated forms of anti-oncogenes. Such inhibition may be provided through recombinant retroviruses carrying a vector construct encoding "inhibitor palliatives," such
30 as: (a) antisense RNA; (b) a mutant protein analogue to a pathogenic protein, which interferes with expression of the pathogenic state; (c) a protein that activates an otherwise inactive precursor; (d) defective interfering structural proteins; (e) peptide inhibitors of viral
35 proteases or enzymes; (f) tumor suppressor genes; or (g) a RNA ribozyme capable of specifically cutting and degrading RNA molecules corresponding to the pathogenic state.

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Alternatively, such inhibition is attained by a recombinant retrovirus capable of site-specific integration into pathogenic genes, thereby disrupting them.

5   Such inhibition may also be accomplished through the expression of a palliative that is toxic for a diseased cell. Where a toxic palliative is to be produced by cells containing the recombinant viral genome, it is important that either the recombinant retrovirus
10   infect only target cells or express the palliative only in target cells, or both. In either case, the final toxic agent is localized to cells in the pathogenic state. Where expression is targeted, the pathogenic agent controlling expression of the toxic palliative could be,
15   for instance, a protein produced through transcription and translation of a pathogenic viral genome present in the cell.

          It should be understood in the foregoing discussion, and throughout this application, that when
20   reference is made to the viral construct "expressing" or "producing" any substance in a cell, or the like, this in fact refers to the action of the resulting provirus following reverse transcription of the viral RNA in the cell. In the context of a toxic palliative, the conse-
25   quent killing effect may not necessarily require the permanent integration of the recombinant viral genome into the host genome, but simply a reasonably long-term expression of a toxic palliative gene, in whatever form desirable, over a reasonably long period of time (several
30   days to one month). Thus, other nonintegrating viral vectors such as, but not limited to, adenoviral vectors may be used for this purpose. Examples of conditional toxic palliatives include recombinant retroviruses encoding (a) a toxic gene product under the control of a
35   cell cycle-specific promoter, a tissue-specific promoter or both; (b) a gene product which is conditionally expressed and which in itself is not toxic but which

processes within target cells a compound or drug from a
nontoxic precursor form to an active toxic form; (c) a
gene product which is not in itself toxic, but when
processed by a protein, such as protease specific to a
5   viral or other pathogen, is converted into a toxic form;
(d) a conditionally expressed reporter gene product on the
cell surface which identifies the pathogenic cells for
attack, for example, by immunotoxins; (e) conditionally
expressed gene products on the cell surface which lead to
10 a toxic effect by interaction with extracellular factors;
and (f) conditionally expressed ribozymes specific for RNA
molecules essential for viability.

Within a related aspect, the present invention
also provides methods for diminishing or eliminating an
15 unwanted or deleterious immune response. Immune suppres-
sion, where appropriate, can be achieved by targeting
expression of immune suppressive genes, such as the
virally derived E3 gene of adenovirus.

Within another aspect of the present invention,
20 methods are disclosed for inhibiting the interaction of
viral particles with cells, cells with cells, or cells
with factors. The methods generally comprise infecting
susceptible cells with a recombinant, replication defec-
tive retrovirus which directs the expression of a blocking
25 element in infected cells, the blocking element being
capable of binding with a cell receptor (preferably the
host cell receptor) either while the receptor is intra-
cellular or on the cell surface, or alternatively, by
binding with the agent. In either event, the interaction
30 is blocked.

Regardless of the means by which the recombinant
retrovirus exerts its immunogenic or inhibitory action as
described above, it is preferred that the retroviral
genome be "replication defective" (i.e., incapable of
35 reproducing in cells infected with it). Thus, there will
be only a single stage of infection in either an _in vitro_
or _in vivo_ application, thereby substantially reducing the

possibility of insertional mutagenesis. Preferably, to assist in this end, the recombinant retrovirus lacks at least one of the gag, pol, or env genes. Further, the recombinant viral vector is preferably chimeric (that is, the gene which is to produce the desired result is from a different source than the remainder of the retrovirus). A chimeric construction further reduces the possibility of recombination events within cells infected with the recombinant retrovirus, which could produce a genome that can generate viral particles.

Within another aspect of the present invention, recombinant retroviruses which are useful in executing the above methods as well as delivering other therapeutic genes are disclosed. The present invention also provides a method for producing such recombinant retroviruses in which the retroviral genome is packaged in a capsid and envelope, preferably through the use of a packaging cell. The packaging cells are provided with viral protein-coding sequences, preferably in the form of two plasmids, which produce all proteins necessary for production of viable retroviral particles, an RNA viral construct which will carry the desired gene, along with a packaging signal which will direct packaging of the RNA into the retroviral particles.

The present invention additionally provides a number of techniques for producing recombinant retroviruses which can facilitate:

i)   the production of higher titres from packaging cells;

ii) packaging of vector constructs by means not involving the use of packaging cells;

iii) the production of recombinant retroviruses which can be targeted for preselected cell lines;

iv) the construction of retroviral vectors with tissue-specific (e.g., tumor) promoters; and

v)   the integration of the proviral construct into a preselected site or sites in a cell's genome.

One technique for producing higher titres from packaging cells takes advantage of our discovery that of the many factors which can limit titre from a packaging cell, one of the most limiting is the level of expression of the packaging proteins, namely, the gag, pol, and env proteins, as well as the level of expression of the retroviral vector RNA from the proviral vector. This technique allows the selection of packaging cells which have higher levels of expression (i.e., produce higher concentrations) of the foregoing packaging proteins and vector construct RNA. More specifically, this technique allows selection of packaging cells which produce high levels of what is referred to herein as a "primary agent," which is either a packaging protein (e.g., gag, pol, or env proteins) or a gene of interest to be carried into the genome of target cells (typically as a vector construct). This is accomplished by providing in packaging cells a genome carrying a gene (the "primary gene") which expresses the primary agent in the packaging cells, along with a selectable gene, preferably downstream from the primary gene. The selectable gene expresses a selectable protein in the packaging cells, preferably one which conveys resistance to an otherwise cytotoxic drug. The cells are then exposed to a selecting agent, preferably the cytotoxic drug, which enables identification of those cells which express the selectable protein at a critical level (i.e., in the case of a cytotoxic drug, by killing those cells which do not produce a level of resistance protein required for survival).

Preferably, in the technique briefly described above, the expressions of both the selectable and primary genes is controlled by the same promoter. In this regard, it may be preferable to utilize a retroviral 5' LTR. In order to maximize titre of a recombinant retrovirus from packaging cells, this technique is first used to select packaging cells expressing high levels of all the required

packaging proteins, and then is used to select which of
these cells, following transfection with the desired
proviral construct, produce the highest titres of the
recombinant retrovirus.

5   Techniques are also provided for packaging of
vector constructs by means not involving the use of
packaging cells. These techniques make use of other
vector systems based on viruses such as other unrelated
retroviruses, baculovirus, adenovirus, or vaccinia virus,
10 preferably adenovirus. These viruses are known to express
relatively high levels of proteins from exogenous genes
provided therein. For such DNA virus vectors, recombinant
DNA viruses can be produced by in vivo recombination in
tissue culture between viral DNA and plasmids carrying
15 retroviral or retroviral vector genes. The resultant DNA
viral vectors carrying either sequences coding for
retroviral proteins or for retroviral vector RNA are
purified into high titre stocks. Alternatively, the
constructs can be constructed in vitro and subsequently
20 transfected into cells which provide in trans viral
functions missing from the DNA vectors. Regardless of the
method of production, high titre (10^7 to 10^{11} units/ml)
stocks can be prepared that will, upon infection of
susceptible cells, cause high level expression of
25 retroviral proteins (such as gag, pol, and env) or RNA
retroviral vector genomes, or both. Infection of cells in
culture with these stocks, singly or in combination, will
lead to high-level production of retroviral vectors, if
the stocks carry the viral protein and viral vector genes.
30 This technique, when used with adenovirus or other
mammalian vectors, allows the use of primary cells (e.g.,
from tissue explants or cells such as WI38 used in
production of vaccines) to produce recombinant retroviral
vectors.

35   In an alternative to the foregoing technique,
recombinant retroviruses are produced by first generating
the gag/pol and env proteins from a cell line infected

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with the appropriate recombinant DNA virus in a manner
similar to the preceding techniques, except that the cell
line is not infected with a DNA virus carrying the vector
construct. Subsequently, the proteins are purified and
5   contacted with the desired viral vector RNA made in vitro,
transfer RNA (tRNA), liposomes, and a cell extract to
process the env protein into the liposomes, such that
recombinant retroviruses carrying the viral vector RNA are
produced. Within this technique, it may be necessary to
10 process the env protein into the liposomes prior to
contacting them with the remainder of the foregoing
mixture. The gag/pol and env proteins may also be made
after plasmid mediated transfection in eukaryotic cells,
in yeast, or in bacteria.

15   The technique for producing recombinant retro-
viruses which can be targeted for preselected cell lines
utilizes recombinant retroviruses having one or more of
the following: an env gene comprised of a cytoplasmic
segment of a first retroviral phenotype, and an extracel-
20 lular binding segment exogenous to the first retroviral
phenotype (this binding segment is from a second viral
phenotype or from another protein with desired binding
properties which is selected to be expressed as a peptide
which will bind to the desired target); another viral
25 envelope protein; another ligand molecule in place of the
normal envelope protein; or another ligand molecule along
with an envelope protein that does not lead to infection
of the target cell type. Preferably, in the technique
briefly described above, an env gene comprised of a
30 cytoplasmic segment of a retroviral phenotype is combined
with an exogenous gene encoding a protein having a
receptor-binding domain to improve the ability of the
recombinant retrovirus to bind specifically to a targeted
cell type, e.g., a tumor cell. In this regard, it may be
35 preferable to utilize a receptor-binding domain which
binds to receptors expressed at high levels on the surface
of the target cell (e.g., growth factor receptors in tumor

cells) or alternatively, a receptor-binding domain binding
to receptors expressed at a relatively higher level in one
tissue cell type (e.g., epithelial cells, ductal
epithelial cells, etc., in breast cancer). Within this
technique, it may be possible to improve and genetically
alter recombinant retroviruses with specificity for a
given tumor by repeated passage of a replicating
recombinant retrovirus in tumor cells; or by linking the
vector construct to a drug resistance gene and selecting
for drug resistance.

The technique for the construction of retroviral
vectors with tissue (e.g., tumor) -specific promoters
utilizes recombinant retroviruses having regulatory
control elements operative in a tissue of interest (e.g.,
beta globin gene promoter in bone marrow leading to
expression in reticulocytes, immunoglobulin promoter in B
cells, etc); the tissue-specific regulatory control
element being able to direct expression of a gene encoding
a lethal agent in target cells in which the control
elements are operable. The operability of the regulatory
control element in different tissues may not need to be
absolutely-specific for a particular tissue to be used in
this technique, since quantitative differences in
operability may be sufficient to confer a substantial
level of tissue specificity to the lethality of the agent
under the control of the element.

Techniques for integrating a retroviral genome
at a specific site in the DNA of a target cell involve the
use of homologous recombination, or alternatively, the use
of a modified integrase enzyme which will recognize a
specific site on the target cell genome. Such site-
specific insertion allows genes to be inserted at sites on
the target cells' DNA, which will minimize the chances of
insertional mutagenesis, minimize interference from other
sequences on the DNA, and allow insertion of sequences at
specific target sites so as to reduce or eliminate the

expression of an undesirable gene (such as a viral gene) in the DNA of the target cell.

It will be appreciated that any of the above-described techniques may be used independently of the others in particular situations, or can be used in conjunction with one or more of the remainder of the techniques.

These and other aspects of the present invention will become evident upon reference to the following detailed description and attached drawings.

Brief Description of the Drawings

Figure 1 depicts three different families of vectors used to produce HIV env and which may or may not have the selectable SV-Neo cassette inserted.

Figure 2 illustrates the HIV env expression levels seen in polyacrylamide gel electrophoresis of HIV env-specific radioimmune precipitations of extracts of human Sup T1 cells transfected with the vectors shown. The markers are in kilodaltons, gp 160 and gp 120 mark the appropriate proteins, and 517 + tat is the positive control (HIV LTR driving env in the presence of tat).

Figure 3 depicts the protocol for testing T-cell killing induced in mice injected with syngeneic tumor cells expressing HIV env (the vector is pAF/Env^r/SV$_2$neo).

Figure 4A graphically depicts the results of the experimental protocol in Figure 3. The specific killing is seen in the top graph with BC10MEenv-29 being killed but not B/C10ME control cells lacking in HIVenv-expression.

Figure 4B illustrates the specificity of the CTL for HIV envelope antigens.

Figure 4C demonstrates the phenotype of the effector cell population generated in the experimental protocol in Figure 3. The effector cell population is that of an L3T4$^-$lyt2$^+$ (CD4$^-$CD8$^+$) T lymphocyte.

Figure 4D illustrates the MHC restriction requirements for the Balb/c anti-BCenv CTL response.

Figure 4E demonstrates that CTL can be induced in vivo by irradiated nonproliferating stimulator cells.

Figure 4F illustrates the dose-response relationship of immunizing Balb/c mice with BCenv stimulator cells.

Figure 4G demonstrates the generation of CTL responses by different H-2^d mouse strains as well as F1 hybrid mice against BCenv target cells.

Figure 4H demonstrates that CTL induced in mice to envelope of the HIV III B strain of virus kill (B/C 10ME env) HIVenv-expressing target cells as well as non-target B/C 10 ME cells (BC) if they are coated with peptide homologous to the HIV III B strain of virus (RP 135). The results also demonstrate that these CTL cross-react and kill some cells coated with peptide homologous to the envelope of the MN strain of HIV (RP 142).

Figure 4I demonstrates induction of HIV envelope specific murine CTL (Env-specific-CTL) following intraperitoneal injection of 10^7 BC cells transfected with plasmid DNA having a recombinant vector construct in which the cytomegalovirus (CMV) promoter directs transient expression of the HIV IIIb envelope as evidenced by specific CTL killing of BCpCMVenv IIIb target cells transiently-expressing HIVenv (BCpCMVenvIIIb target) and cloned stable - HIVenv-expressing BC29 target cells (BC29 target) but not non-infected BC cells (BC10ME target).

Figure 4J demonstrates that murine BC target cells infected two days before use by transfection with CMVH2-Dd or RSV-Dd which encode H2-Dd under control of the Cmv or RSV-LTR promoter, respectively, serve as target cells for killing by C57BL16 muring CTL immunized with Balb/C cells (H2-Dd). These CTL specifically kill target cells expressing H2-Dd but not control 3T3 cells.

Figure 4K graphically depicts the results of an experimental protocol similar to that in Figure 3, wherein

mice were injected with cells expressing gag/pol (BC-1-16H) or gag/prot (BC-1) instead of cells expressing HIVenv. CTL from gag/pol or gag/prot immunized mice killed their respective target cell, either BC-1 or BC-1-16H target cells, respectively, but not B/C 10 ME cells.

Figure 4L demonstrates that CTL induced by gag/pol stimulator cells (BC-1-15H; abbreviated 1-15H) killed both gag/pol (1-15H) and gag/prot (BC-1) target cells.

Figure 4M demonstrates that CTL induced by gag/prot stimulator cells (BC-1) killed both gag/prot (BC-1) and gag/pol (1-15H) target cells.

Figure 4N demonstrates that two (2x) direct injections of HIVenv encoding retrovirus by the intraperitoneal (I.P.) or intramuscular (I.M.) routes stimulated the development of CTL capable of specifically killing BC10MEenv-29 cells but not B/C 10 ME control cells (BC)

Figure 4O demonstrates _in_ _vitro_ immunization of human donor #99PBL using autologous EBV-transformed stimulator cells from donor #99 (99-EBV) that were infected _in_ _vitro_ with recombinant retroviral vector encoding HIVenv (99-EBV-HIVenv) to effect induction of CTL which kill 99-EBV-HIVenv target cells but not negative control autologous cells, i.e., 99-PHA stimulated PBL (99 PHA blasts). Donor #99 expressed natural immunity to EBV, as evidenced by a lower level of killing of positive control 99-EBV target cells.

Figure P demonstrates that human HT1080 cells infected with both murine MHC H2-Dd and HIVenv antigen (HT1080 + Dd + env) simultaneously express both gene products in a functionally-active form as evidenced by the lysis of these cells by HIVenv-immune murine CTL. These HIVenv-immune CTL did not induce significant lysis of either control HT1080 (HT1080) cells or BC cells expressing H2-Dd (BC-Dd).

19

Figure 4Q demonstrates in panel A the lysis of BC cells expressing HIV envelope proteins (BCenvIIIb) and BC cells coated with RP135 "V3loop" ("loop") synthetic peptide by HIVenv-immune CTL; in panel B, the lysis of BCenvΔV3 cells lacking the gp120 hypervariable envelope loop (i.e., "loopless") by CTL immunized with "loopless" BCenvΔV3 cells. The CTL immunized with "loopless" BCenvΔV3 were specific, i.e., they did not lyse BC cells lacking in HIVenv (BC) and they did not lyse cells coated with the "loop" RP135 peptide (BC-RP135).

Figure 4R demonstrates: in panel A, the specific lysis of BC cells expressing a truncated envelope protein from a recombinant HIVenv gene (BCpcmv Chunk 1 target; referred to as "Chunk 1" (see Example 1F2)), and also cloned infected BC cells expressing HIVenvIIIb (BCenvIIIb 29 target) by BCenvIIIb 29-immune murine CTL; and in panel B, the induction of immune CTL in mice immunized with BCpCMVChunk 1 cells which specifically kill BCpCMVChunk 1 target cells and BCenvIIIb-14H target cells but not non-infected BC cells (BC10ME target).

Figure 5 depicts a vector designed to express sCD4.

Figure 6 illustrates the construction of the plasmids carrying the vectors TK1 (without SV-Neo) and TK3 (plus SV-Neo).

Figure 7 illustrates the construction of the plasmid carrying the vector KTVIHAX.

Figure 8 illustrates the construction of the plasmids carrying the vectors KTVIH5 (without SV-Neo) and KTVIH Neo (with SV-Neo).

Figure 9 illustrates construction of the plasmid carrying the vector MHMTK-Neo.

Figure 10 illustrates the construction of the plasmid carrying the vector RRKTVIH.

Figure 11 illustrates the construction of the plasmids carrying the tat-his (tat in sense direction) or αtat (tat in antisense direction) vectors.

Figure 12 graphically depicts the preferential killing of PA317 cells infected with tathis vector (5 clones, TH1-5) compared to control PA317, upon infection with the three conditional lethal vectors shown and
5   treatment with acyclovir (ACV).

Figure 13 illustrates the constuction of the plasmid carrying the vector 4TVIHAX.

Figure 14 depicts the construction of a viral vector carrying HIV inducible marker/reporter genes such
10   as alkaline phosphatase (AP).

Figure 15 depicts the structure of an HIV inducible marker/reporter gene carried on a plasmid which can be transfected into cells.

Figure 16 graphically depicts a time course of
15   HIV infection of Sup T1 cells carrying the AP marker in Figure 15 with HIV at various concentrations of AZT. The level of HIV infection was measured by taking small aliquots of supernatant.

Figure 17 graphically depicts the results of the
20   same experiment as in Figure 16, but with ddC as the HIV inhibitor.

Figure 18 diagrammatically illustrates the number of cells surviving after phleomycin selection upon transfection of cells with a plasmid which expresses the
25   phlemoycin resistance gene (PRG) directly from a promoter (right, complete line), and with another which expresses PRG with a coding sequence interposed between it and the promoter (left, dotted line).

Figure 19 depicts four plasmids designed to
30   express retroviral proteins in mammalian cells. pSVgp and pRSVenv are cotransfected with a selectable marker, while pSVgp-DHFR and pRSVenv-phleo are the equivalent plasmids with the selectable marker placed downstream of the viral protein-coding regions.

35   Figure 20 depicts three sites of fusion of HIV env and MoMLV env after site-directed mutagenesis. The joint at the extracellular margin of the transmembrane

region is designated as A, while B and C indicate loca-
tions of joints at the middle of the transmembrane region
and cytoplasmic margin, respectively. The numbering is
according to nucleotide numbers (RNA Tumor Viruses, Vol.
5   II, Cold Spring Harbor, 1985). ST, SR, SE are the starts
of tat, rev and env while TT, TR, and TE are the
corresponding termination sites.

            Figure 21 depicts the substitution of U3 in a 5'
LTR by a heterologous promoter/enhancer which can be fused
10 to either the Sac I, Bssh II or other site in the region.

            Figure 22 illustrates a representative method
for crossing transgenic mice expressing viral protein or
vector RNA.

15 Detailed Description of the Invention

    I.   Immunostimulation

            The ability to recognize and defend against
foreign pathogens is central to the function of the immune
20 system. This system, through immune recognition, must be
capable of distinguishing "self" from "nonself" (foreign),
which is essential to ensure that defensive mechanisms are
directed toward invading entities rather than against host
tissues. The fundamental features of the immune system
25 are the presence of highly polymorphic cell surface
recognition structures (receptors) and effector mechanisms
(antibodies and cytolytic cells) for the destruction of
invading pathogens.

            Cytolytic T lymphocytes (CTL) are normally
30 induced by the display of processed pathogen-specific
peptides in conjunction with the MHC class I or class II
cell surface proteins. Also stimulated by this type of
antigen presentation are the generation and production
antibodies, helper cells and memory cells. Within one
35 embodiment of the present invention, presentation of
immunogenic viral determinants in the context of appro-
priate MHC molecules efficiently induces optimal CTL

responses without exposing the patient to the pathogen.
This vector approach to immunostimulation provides a more
effective means of inducing potent class I-restricted
protective and therapeutic CTL responses, because the type

5   of immunity induced by the vector more closely resembles
that induced by exposure to natural infection. Based on
current knowledge of several viral systems, it is unlikely
that exogenously supplied, nonreplicating viral antigens,
such as peptides and purified recombinant proteins, will

10   provide sufficient stimulus to induce optimal class I-
restricted CTL responses. Alternatively, vector-delivered
expression of selected viral proteins or other antigens
corresponding to a pathogenic condition, such as cancer,
within target cells as described within the present

15   invention provides such a stimulus.

By way of example, in the case of HIV-1
infections, patients develop antibodies specific for a
variety of viral envelope-region determinants, some of
which are capable of _in vitro_ virus neutralization.

20   Nevertheless, disease progression continues and the
patients eventually succumb to the disease. Low-level CTL
responses against infected patients' cells (Plata et al.,
Nature 328:348-351, 1987) and against target cells
infected with recombinant vaccinia vectors expressing HIV

25   gag, pol, or env (Walker et al., _Nature_ 328:345-348, 1987;
Walker et al., _Science_ 240:64-66, 1988) have been detected
in some HIV-1 seropositive patients. In addition, it has
recently been shown that murine as well as human CTL can
be induced by autologous stimulator cells expressing HIV

30   gp 120 via transfection (Langlade-Demoyan et al.,
J. Immunol. 141:1949, 1988). Improved CTL induction could
be therapeutically advantageous to infected patients and
provide effective preventive therapy to individuals under
noninfectious conditions. HIV infection itself may not be

35   producing an adequate CTL response because other elements
associated with HIV infection may prevent proper immune
stimulation. In addition, it may be that stimulation of

T-cells by infected cells is an interaction that leads to
infection of the stimulated T-cells.

HIV is only one example. This approach should
be effective against many virally linked diseases or
5 cancers where a characteristic antigen (which does not
need to be a membrane protein) is expressed, such as in
HPV and cervical carcinoma, HTLV-I-induced leukemias,
prostate-specific antigen (PSA) and prostate cancer,
mutated p53 and colon carcinoma, GD2 antigen and melanoma.
10 Example 1 describes procedures for constructing plasmids
capable of generating retroviral vectors in packaging
cells, which then lead to expression of HIV viral
antigens.

15   EXAMPLE 1
                 Vectors Expressing HIV Antigens
      A.   Env Expression Vector (See Figure 1):
           A 2.7 kb Kpn-Xho I DNA fragment was isolated
from the HIV proviral clone BH10-R3 (for sequence, see
20 Ratner et al., Nature 313:277, 1985) and a ≈400 bp Sal-
Kpn I DNA fragment from IIIexE7deltaenv (a Bal31 deletion
to nt. 5496) was ligated into the Sal I site in the
plasmid SK+. From this clone, a 3.1 kb env DNA fragment
(Xho I-Cla I) which also encodes rev, essential for env
25 expression, was purified and ligated into a retroviral
vector called pAFVXM (see Kriegler et al., Cell 38:483,
1984). This vector was modified in that the Bgl II site
was changed by linker insertion to a Xho I site to
facilitate cloning of the HIV env coding DNA fragment.
30   A dominant selectable marker gene comprised of a
SV40 early promoter driving expression of neomycin
phosphotransferase gene was inserted into the vector at
the Cla I site to facilitate isolation of infected and
transfected cell lines. This vector is called
35 pAF/Env⁻/SV₂neo (see Figure 1).
           The Xho I site upstream from the ENV gene in the
vector provides a convenient site to insert additional

promoters into the vector construct as the RSV promoter,
SV40 early or late promoter, the CMV immediate early (IE)
promoter, human beta-actin promoter, and Moloney murine
MLV SL3-3 promoter.

5   One such promoter, the CMV Immediate Early gene
promoter (see Figure 1), a 673 bp DNA fragment Hinc II to
Eag I, results in a tenfold increase in ENV expression in
a human T-cell line called Sup T1 when compared to the
parental construct pAF/Env^r/SV$_2$neo (see Figure 2).

10   To improve titres of the vector one can use a
recombinant retrovirus based on N2 (Armentano et al., J.
Virol. 61:1647-1650, 1987; Eglitas et al., Science
230:1395-1398, 1985). This vector contains both the
packaging sequences from N2 as well as the bacterial
15 neomycin phosphotransferase gene. The above HIV env
construct was inserted into the unique Xho I site in N2 as
follows.

          The MoMuLV 5' LTR fragment, including GAG
sequences, from N2 (Eco RI-Eco RI) was ligated into
20 plasmid SK$^+$ resulting in a construct called N2R5. The
N2R5 construct was mutagenized by site-directed in vitro
mutagenesis to change the GAG ATG to ATT. This
mutagenized site was flanked by Pst I sites 200 bp apart.
The 200 bp mutagenized fragment was purified and inserted
25 into the same Pst I sites (to replace the non-mutagenized
200 bp fragment) of N2 MoMuLV 5' LTR in plasmid pUC 31.
The resulting construct was called pUC 31/N2R5 g^M. (pUC31
was derived from pUC19, with additional Xho I, Bgl II, Bss
HII and Nco I sites inserted between the Eco RI and Sac I
30 sites of the polylinker). The 1.0 kb MoMuLV 3' LTR
fragment from N2 (Eco RI-Eco RI) was cloned into plasmid
SK$^+$ resulting in a construct called N2R3(-).

          From the vector pAF/Env^{r}SV$_2$neo the 3.1 kb env
DNA fragment (Xho I-Cla I) which also encodes rev,
35 essential for env expression, was purified. From the
plasmid N2R3(-), the 1.0 kb MoMuLV 3' LTR fragment (Cla I-
Hind III) was purified.

The N2-based env expression vector was produced
by a three-part ligation in which the 3.1 kb env fragment
(Xho I-Cla I) and the 1.0 kb MoMuLV 3' LTR fragment (Cla
I-Hind III) were inserted into Xho I-Cla I site of pUC
5   31/N2R5 g^M.

A dominant selectable marker gene comprised of a
SV40 early promoter driving expression of the neomycin
phosphotransferase gene was inserted into the N2-based env
expression vector at the Cla I site to facilitate
10   isolation of infected and transfected cell lines. This
vector was called KT-1.

B.   Gag Expression Vector:

To efficiently express HIV gag and pol gene
products in a retrovirus vector, two criteria must be met:
15   1) a REV response element (RRE) must be added to the
vector to override repressive elements buried in gag and
pol; and 2) REV must be efficiently expressed to interact
with the RRE inserted in the vector, thus allowing for
correct transport of viral messenger RNA into the
20   cytoplasm.

A 2.5 kb Sac I-Eco RV DNA fragment was isolated
from pBH10-R3 (see Ratner et al., op. cit.) and ligated
into the Sac I-Sma I site of pUC31 along with a linker
coding for a universal translation termination codon.
25   pUC31 is derived from pUC19 with additional Xho I, Bgl II,
Bss HII and Nco I sites inserted between the Eco Rl and
Kpn I sites of the poly linker. However, this construct
contained the major splice donor (SD) site from HIV and
thus could be problematic in virus generation. The SD
30   site was removed by subcloning a 70 bp Rsa I-Cla I
fragment with a 2.1 kb Cla I-Bam HI DNA fragment into the
Hinc II-Bam HI site of SK$^+$. The Bam HI site was converted
into a Cla I site by linker insertion. This construct was
designated SK$^+$ gag protease SD delta.

35   A gag/pol SD deletion complete construct was
produced by a three-part ligation reaction in which a 757
bp Xho-Spe I fragment from SK$^+$ gag protease SD delta and a

4.3 kb Spe I-Nco I fragment from BH10 R3 were inserted into SK⁺ XhoI-Nco I. The Xba I site in SK⁺ was converted to a Nco I to facilitate this reaction.

In order to introduce both REV and the REV
5 responsive elements in the vector, a 1.4 kb Ssp I deletion in the plasmid SK⁺ HIV env was generated. This deletion removed intronic sequences which are not important for REV expression (REV expression will continue to be from a spliced mRNA.) In addition, this deletion does not effect
10 the REV responsive element located in env. The 1.1 kb DNA fragment coding for the dominant selectable marker Neo, engineered to contain a eukaryotic translation initiation codon, was introduced into the construct at the Bgl II site in env. Insertion of neo facilitates detection of
15 passaged virus as well as selection for virus in an unspliced state during passage. A promoter such as the CMV is inserted into the XhoI site of this construct. This construct is designated SK⁺ CMV/REV/Neo. The final viral construct may be produced by a four-part ligation
20 reaction. A 2.5 kb Xho I-Xba I DNA fragment from SK⁺ gag polymerase SD delta, a 3.5 kb Spe I-Cla I DNA fragment from SK⁺ CMV/REV/Neo and a 1.2 kb Cla I-Hind III DNA fragment from N2R3(-) (a subclone of N2 containing only the 3' LTR) are inserted into pUC N2R5 (a subclone of N₂
25 containing the 5' LTR) at the Xho I-Hind III site of this construct.

C.   Gag-Pol Expression Using N2-Based Vector:

Efficient expression of HIV gag-pol gene
30 products requires a REV response element (RRE) and REV (as discussed above, see "Gag Expression Vector").

To obtain REV and RRE, a 2.7 kb Kpn I-Xho I DNA fragment was isolated from the HIV proviral clone BH10-R3 (for sequence, see Ratner et al., Nature 313:277, 1985)
35 and a 400 bp Sal I-Kpn I DNA fragment from exE7deltaenv (a Bal 31 deletion to nt 5496) was ligated into the Sal I site in the plasmid SK⁺. This construct was called SK⁺

env^r. A 239 bp 5'REV DNA fragment (Xho I-Ssp I) and a 4.2 kb RRE/3'REV in SK$^+$ fragment (Xho-I-Bgl II) were isolated from SK$^+$ env^r.

To obtain the gag-pol gene, a 2.5 kb Sac I-Eco RI DNA fragment was isolated from pBH10-R3 (see Ratner et al., supra) and ligated into the Sac I-Sma I site of pUC31 along with a linker coding for a universal translation termination codon. However, this construct contained the major splice donor (SD) site from HIV and thus could be problematic in virus generation. The SD site was removed by subcloning a 70 bp RSA I-Cla I fragment with a 2.1 kb Cla I-Bam HI DNA fragment into the Hind II-Bam HI site of SK$^+$. The Bam HI site was converted into a Cla I site by linker insertion. This construct was designated SK$^+$ gag protease SD delta.

A gag-pol SD deletion complete construct was produced by a three-part ligation in which a 757 bp Xho I-Spe I fragment from SK$^+$ gag protease SD delta and a 4.3 kb Spe I-Nco I fragment from BH10-R3 were inserted into SK$^+$ Xho I-Nco I. The Xba I site in SK$^+$ was converted to a Nco I to facilitate this reaction. In addition, the Nde 1 site in pol was converted to an Xba I site. The resulting construct was called SK$^+$ gag-pol SD delta. The Xba I site from this construct was again converted to create a Bam HI site, and the 4.2 kb gag-pol DNA fragment (Xho I/blunt-Bam HI) was isolated.

The SK$^+$ gag-pol expression vector was produced by a three-part ligation in which the 239 bp 5'REV DNA fragment (Xho-I-Ssp I) and the 4.2 kb gag-pol DNA fragment (Xho I/blunt-Bam HI) were inserted into the Xho I-Bgl II 4.2 kb RRE/3'REV in SK$^+$ vector fragment. The resulting construct was called SK$^+$ gag-pol/RRE/REV.

The N2-based gag-pol expression vector was produced by a two-part ligation in which the 5.7 kb gag-pol/RRE/REV fragment (Xho I-Cla I), from SK$^+$ gag-pol/RRE/REV, was inserted into the Xho I-Cla I site of pUC31/N2R5 g^M.

A dominant selectable marker gene fragment from N2 (EcoRI-EcoRI comprised of a SV40 early promoter driving expression of the neomycin phosphotransferase gene was cloned into plasmid SK⁺. From this a 1.3 kb neo gene

5   fragment (Cla1-Bst B1) was inserted into the Cla I site of the N2-based gag-pol expression vector to facilitate isolation of infected and transfected cell lines. This vector was called KT-2

10   D.   <u>Gag-Protease -RT Expression Using N2-based Vector</u>

Efficient expression of gag-protease-reverse transcriptase (gag-protease-RT) gene products (gag/prot) requires RRE and REV (as discussed above, see "Gag Expression Vector").

15   REV and RRE were obtained as described above (see "Gag-pol Expression Using N2-based Vector").

The gag gene contains a major splice donor (SD) site which could be a problem in virus generation. The SD site was removed by changing GT to AC (nt 744,745) by

20   site-directed <u>in vitro</u> mutagenesis of pSLCATdelBgl II (a vector that expresses gag-pol, tat, and rev, derived from HIV strain HXB2). A Sac I site was also created upstream of the SD delta site so that a 780 bp SD delta gag fragment (Sac I-Spe I) could be purified. A 1.5 kb gag-

25   prot-RT fragment (Spe I-EcoRV) and the 780 bp SD delta gag fragment (Sac I-Spe I) were inserted into pUC18 (Sac I-Sma I). The resulting 2.3 kb SD delta gag-prot-RT fragment (Sac I/blunt-Bam HI) was isolated from this pUC18 vector.

The SK⁺ gag-prot-RT expression vector was

30   produced by a three-part ligation in which the 239 bp 5'REV DNA fragment (Xho I-Ssp I) and the 2.3 kb SD delta gag-prot-RT fragment (Sac I/blunt-Bam HI) were inserted into the Xho I-Bgl II 4.2 kb RRE/3'REV in SK⁺ vector fragment. The resulting construct was called SK⁺ gag-

35   prot-RT/RRE/REV.

The N2-based gag-prot-RT expression vector was produced by a two-part ligation in which the 3.8 kb gag-

prot-RT/RRE/REV fragment (Xho I-Cla I), from SK⁺ gag-prot-RT-RRE/REV, was inserted into the Xho I-Cla I site of pUC31/N2R5 g^M.

A dominant selectable marker gene fragment from N2 (EcoRA-EcoR1), comprised of a SV40 early promoter driving expression of the neomycin phosphotransferase gene, was cloned into plasmid SK⁺. From this, a 1.3 Kb neo gene fragment (ClaI-Bst BI) was inserted into the N2-based gag-pol-RT expression vector at the Cla I site to facilitate isolation of infected and transfected cell lines. This vector was called KT-3.

E.   Construction of H2-Dd expression vectors

The murine class I gene encoding H2-Dd gene was cloned into a Bluescript™ SK⁺ plasmid containing either the CMV promoter or the RSV LTR inserted upstream of the H2-Dd gene. The expression constructs RSV-Dd and CMV-Dd were transfected into 3T3 cells using the CaPO₄/polybrene method.

The expression constructs CMV-Dd and MV7.T4 (a retroviral construct expressing human CD4 and neomycin phosphotransferase) were co-transfected into human HT1080 cells. The cells were selected with G418 and resistant colonies were picked, expanded and tested for expression of H2-Dd by Western blot using a rat anti-Dd antibody. One of the positive clones (A-9) was infected with N2env-neo (KT-1).

F.   Construction of HIV envelope deletions for CTL epitope mapping:

Two retroviral vectors were prepared for mapping the regions in the HIV envelope proteins which contain peptide epitopes reactive with CTL. The first, referred to as Δloop, contains a deletion of the gp120 hypervariable loop which is a region of the HIV IIIB envelope protein reactive with antibody in patient's sera. The second, referred to as "Chunk I", contains only the envelope region of HIV IIIB envelope from the amino

terminal up to the beginning of the gp120 loop and also
lacks the membrane gp41 "tail" of the envelope protein.

1.   A Loop

        A 602 base pair Hinc II-Sca I fragment of HIV
5   IIB env was prepared and inserted into plasmid 130B.   A
43-mer oligo 5' G AAC CAA TCT GTA GAA ATT AAT AAC AAT AGT
AGA GCA AAA TGG 3' was synthesized.   Mutagenesis of the
mismatched primer was done by the method of Kunkel
(Kunkel, T. A. Proc. Natl. Acad. Sci. USA 82:448-493,
10 1985) to generate an in frame deletion of 106 amino acids.
This was confirmed by DNA sequencing.   The Stu I-Aoc I
fragment containing the deletion was then inserted into
the same sites of HIV IIIb env that was carried in
Bluescript SK+ (Stratagene, La Jolla).   From this
15 intermediate, the Xho I-Cla I fragment of HIV env IIIB
containing the deletion was inserted into the same sites
of a modified N2 retroviral vector.   (This vector did not
have the gag ATG start codon.)   The SV2neo gene was lastly
inserted into the Cla I site in the sense orientation.

20 2.   Chunk I

        The 1.30 kb Xho I-PVU II fragment of HIV IIIB
env was cloned into the Bluescript SK+ vector (Stratagene,
La Jolla) at the Xho I-Hinc II sites.   This fragment was
then reisolated from the vector as Xho I-Bam HI fragment
25 (Bam HI site from the polylinker) and inserted into the
Xho I-Bgl II sites of HIV env IIIB carried either in the
KT-1 retroviral vector or a CMV construct expressing HIV
IIIB env.

        These plasmids, when placed in a suitable
30 packaging cell, expressed a retroviral vector construct
which contains a packaging signal.   The packaging signal
directed packaging of the vector construct into a capsid
and envelope along with all further proteins required for
viable retroviral particles.   The capsid, envelope, and
35 other proteins are preferably produced from one or more
plasmids containing suitable genomes placed in the
packaging cell.   Such genomes may be proviral constructs,

which in a simple case may merely have the packaging
signal deleted. As a result, only the vector will be
packaged. Suitable packaging or packaging cell lines, and
the genome necessary for accomplishing such packaging, are
5   described in Miller et al. (Mol. Cell. Bio. 6:2895, 1986),
which is incorporated herein by reference. As described
by Miller et al., it is preferable that further changes be
made to the proviral construct other than simple deletion
of the packaging signal in order to reduce the chances of
10 recombination events occurring within the packaging cell
line, which may result in production of viral particles
which are not replication defective.

It will be understood that Example 1 is merely
illustrative of a procedure for generating an HIV envelope
15 glycoprotein (gp) or other viral antigen. It is also
possible to provide a proviral vector construct which
expresses a modified HIV envelope gp on the target cells
which will likewise stimulate an immune response, but with
less T-cell cytopathic effects. Envelope glycoproteins
20 can be suitably modified using techniques well known in
the art, for instance through use of the disclosure of
articles such as Kowalski et al. (Science 237:1351, 1987),
which is herein incorporated by reference. Thus, a
proviral construct may be constructed by the above
25 technique which generates retroviral constructs expressing
such a suitably modified gp. This construct is then
placed in a packaging cell as described above. The
resulting recombinant retroviruses produced from the
packaging cell lines may be used in vitro and in vivo to
30 stimulate an immune response through the infection of
susceptible target cells. The nucleic acids introduced by
these means into the susceptible target cell may become
integrated into the nucleic acid of the target cell. It
will be appreciated that other proteins expressed from the
35 HIV genome, such as gag, pol, vif, nef, etc., may also
elicit beneficial cellular responses in HIV-infected
individuals. Proviral vectors such as those described

below are designed to express such proteins so as to
encourage a clinically beneficial immune response. It may
be necessary for certain vectors to include rev coding
sequences as well as a rev responsive element (Rosen
5   et al., Proc. Natl. Acad. Sci. 85:2071, 1988).
        The following example demonstrates the ability
of this type of treatment to elicit CTL responses in mice.

                         EXAMPLE 2

10
A.   Immune Response to Retroviral Vector-Encoded Antigens
        A murine tumor cell line (B/C10ME) (H-2^d) (Patek
et al., Cell. Immunol. 72:113, 1982) was infected with a
recombinant retrovirus carrying the pAF/Env^r/SV$_2$neo vector
15   construct coding for HIV env. One cloned HIV-env
expressing cell line (B/C10ME-29) was then utilized to
stimulate HIV-env-specific CTL in syngeneic (i.e., MHC
identical) Balb/c (H-2^d) mice (see Figure 3). Mice were
immunized by intraperitoneal injection with B/C10ME-29
20   cells (1 x 10^7 cells) and boosted on day 7-14. (Boosting
was not absolutely required.) Responder spleen cell
suspensions were prepared from these immunized mice and
the cells cultured in vitro for 4 days in the presence of
either B/C10ME-29 (BCenv) or B/C10ME (BC) mitomycin-C-
25   treated cells at a stimulator:responder cell ratio of 1:50
(Figure 3). The effector cells were harvested from these
cultures, counted, and mixed with radiolabeled (^{51}Cr)
target cells (i.e., B/C10MEenv-29 or B/C10ME) at various
effector:target (E:T) cell ratios in a standard 4-5 hr
30   ^{51}Cr-release assay. Following incubation, the microtiter
plates were centrifuged, 100 ul of culture supernate was
removed, and the amount of radiolabel released from lysed
cells quantitated in a Beckman gamma spectrometer. Target
cell lysis was calculated as: % Target Lysis = Exp CPM -
35   SR CPM/MR CPM - SR CPM x 100, where experimental counts
per minute (Exp CPM) represents effectors plus targets;
spontaneous release (SR) CPM represents targets alone; and

33

maximum release (MR) CPM represents targets in the presence of 1M HCl.

The results (Figure 4A) illustrate that CTL effectors were induced which specifically lysed HIV-env-expressing target cells (BCenv) significantly more efficiently than non-HIV env BC targets. Primed spleen cells restimulated in vitro with non-HIV-env-expressing control cells (B/C10ME) did not show significant CTL activity on either B/C10MEenv-29 or B/C10ME targets, particularly at lower E:T cell ratios. Spleen cells obtained from naive nonimmunized Balb/c mice which were stimulated in vitro with B/C10MEenv-29 did not generate CTL (data not shown), thus suggesting the importance of the in vivo priming and boosting event. This experiment has been repeated and similar results obtained.

In another experiment, effector cells obtained from Balb/c mice immunized, boosted and restimulated in vitro with a different H-2^d HIV-env-expressing tumor cell clone (L33-41) infected with the same pAF/Env^r/SV$_2$neo (HIV-env) vector construct were capable of lysing B/C10MEenv-29 target cells. This provides additional support that the CTL generated in these mice are specifically recognizing an expressed form of HIV-env rather than simply a unique tumor cell antigen on these cells. This result also suggests that the vector-delivered antigen is presented in a similar manner by the two tumor cell lines. The specificity of the CTL response was further demonstrated by testing effector cells obtained from BCenv immunized mice on BCenv target cells expressing the neo and HIV env genes, BC (non-neo, non-HIV env) parental targets and BCneo target cells expressing the neo resistance marker gene, but no HIV env. Figure 4B indicates that the CTL responses are specific for the HIV env protein.

In another experiment, effector cells obtained from mice immunized with 1 X 10^7 BCenv cells, boosted and restimulated in vitro, were treated with T-cell-specific

monoclonal antibodies (Mab) plus complement (C') in order
to determine the phenotype of the induced cytotoxic
effector cells. Effectors were treated with either anti-
Thy 1.2 (CD3), anti-L3T4 (CD4; RL172.4, Ceredig et al.,
5   *Nature* 314:98, 1985) or anti-Lyt 2.2 (CD8) Mab for 30
minutes at 4°C, washed 1 time in Hank's balanced salt
solution (HBSS), resuspended in low tox rabbit C' and
incubated 30 minutes at 37°C. The treated cells were
washed 3 times in RPMI 1640 complete medium, counted, and
10   tested for their ability to lyse BCenv radio-labeled
target cells as previously described. Figure 4C shows
that treatment with either anti-Thy 1.2 or anti-Lyt 2.2
Mab + C' abrogated cytotoxic activity, whereas treatment
with anti-L3T4 Mab + C' or C' alone did not significantly
15   affect cytotoxicity. These results indicate that the
majority population of cytotoxic effector cells generated
in this system are of the CD3$^+$ CD4$^-$ CD8$^+$ cytotoxic T-cell
phenotype.

         Experiments were performed to determine the MHC
20   restriction of CTL effector cells described above.
Polyclonal antibodies directed against different H-2
regions of the murine MHC (i.e., anti-H-2^d, anti-H-2D^d,
anti-H-2L^d, anti-H-2K^d, anti-H-2I^d) were used to inhibit
the CTL response on BCenv target cells. The anti-H-2^k
25   antiserum was used as a negative control. The data
(Figure 4D) indicate that the Balb/c anti-BCenv CTL
response is inhibited primarily by the anti-H-2D^d
antiserum. This suggests that these CTL responses are
restricted by MHC class I molecules, most likely encoded
30   within the D region of the H-2 complex.

         In addition to experiments in which mice were
immunized with replication-competent HIV env-expressing
tumor cells, tests were conducted to determine whether
proliferating stimulator cells were necessary for inducing
35   CTL *in vivo*. Mice were immunized with either irradiated
(10,000 rads) or nonirradiated BCenv cells, and the primed
spleen cells were later stimulated *in vitro*, as previously

described.   The resulting effector cells were tested for
CTL activity on radiolabeled BCenv and BC target cells.
Figure 4E indicates HIV-specific CTL can be induced _in_
vivo with either irradiated or nonirradiated stimulator
5   cells.   These data demonstrate that CTL induction by HIV
env-expressing stimulator cells is not dependent upon
proliferation of stimulator cells _in vivo_ and that the
presentation of HIV env antigen in the appropriate MHC
context is sufficient for effective CTL induction.
10   Formalin fixed cells also elicit an equivalent immune
response.   This shows that killed cells or perhaps cell
membranes expressing the appropriate antigen in the proper
MHC class I/II molecular context are sufficient for
induction of effective CTL responses.
15   Additional experiments were performed to examine
the optimal injection dose of BCenv cells into Balb/c
mice.   Mice were immunized with varying numbers of BCenv
stimulator cells, restimulated _in vitro_ as described, and
tested for CTL activity.   The results shown in Figure 4F
20   indicate that immunization of mice with 5 X 10^6 env-
expressing BCenv-29 stimulator cells generated an optimal
CTL response under these conditions.
             Further experiments examined the ability of
vector-infected HIV env-expressing BCenv stimulator cells
25   to induce CTL responses in other $H-2^d$ mouse strains other
than Balb/c, in order to provide an indication as to
genetic restrictions imposed on host responsiveness.
Different strains of $H-2^d$ (i.e., Balb/c, DBA/2, B10.D2),
as well as $H-2^d$ x $H-2^b$ F1 hybrid mice [i.e., CB6F1 (Balb/c
30   x B6 F1); B6D2F1 (B6 x DBA/2 F1)], were immunized with
BCenv stimulator cells and examined for the induction of
CTL responses.   Figure 4G illustrates that all strains
including F1 hybrids generate CTL responses against the
BCenv target cells to varying degrees.   Although some
35   strains also exhibit responses against the parental (i.e.,
non-HIV env) target cells, these responses are lower than
those directed against the BCenv target.

36

Experiments were also conducted to evaluate the
ability of the vector-infected HIVenv (strain III B)-
expressing BCenv stimulator cells to induce cross-reactive
CTL responses against other strains of HIV (i.e., MN).
5   Figure 4H illustrates that CTL induced with the HIV env
III B strain of vector-infected BC stimulator cells killed
BC target cells coated with RP135 peptide (Javaherian
et al., Proc. Natl. Acad. Sci. USA 86:6768, 1989)
homologous with the IIIB envelope sequence. These CTL
10 also killed BC target cells coated with RP142 (i.e.,
peptide homologous with the HIV MN strain of envelope
proteins) (Javaherian et al., Proc. Natl. Acad. Sci. USA
86:6768, 1989).

Additional experiments were conducted to examine
15 the ability of transiently-HIVenv expressing stimulator
cells to induce CTL responses in mice. These cells are
distinguished from the BCenv stimulator cells, described
above, by virtue of being infected with retroviral vector
only two days prior to use and not being selected or
20 cloned in vitro prior to use as stimulator cells, i.e., by
injection into mice. The results presented in Figure 4I
illustrate that BC cells transfected two days before
injection into mice with plasmid DNA having the HIVenv
IIIb vector construct (in which the cmv promoter drives
25 expression of HIV envelope protein; BCpcmvIIIb) induced
specific CTL which could be restimulated with BCpcmvIIIb
cells in vitro and would kill BCpcmvIIIb transiently-
expressing target cells (BCpcmvenv IIIB target) as well as
retrovirus-infected BC cells from a cell line which was
30 cloned and selected for stable expression of HIVenvIIIb
(BC-29 target). These specific immune CTL did not kill
BC10ME control cells which do not express HIV envelope
proteins. Thus, B/C cells transiently-expressing HIV
envelope proteins can serve as effective inducers of
35 immune CTL responses in vitro, restimulators of immune CTL
in vitro (i.e., for use in CTL assays, etc.), and target
cells for lysis by immune CTL in vitro. In addition, the

results of similar experiments are presented graphically
in Figure 4J which illustrates that BC cells transiently-
expressing H2-Dd serve as target cells for lysis by H2-Dd
alloimmune CTL.

5   To evaluate the ability of other retroviral
vector-encoded HIV antigens to induce an immune response
in mice, experiments were also conducted with retroviral
constructs encoding gag/pol and gag/prot. The results
presented in Figure 4K illustrates that murine CTL are
10 induced by immunization with either gag/pol or gag/prot
wich can kill their respective target cells, i.e., either
BC-1-16H cells (i.e., infected with the gag/pol vector) or
BC 1 cells (i.e., infected with the gag/prot vector).
          Further experiments were conducted to evaluate
15 the specificity of the CTL killing induced by gag/pol and
gag/prot stimulator cells. The results presented in
Figures 4L and 4M illustrate that CTL induced by gag/pol
or gag/prot stimulator cells killed both gag/pol (i.e., 1-
15H) and gag/prot (i.e., BC-1) target cells. In this
20 case, the killing by CTL may be directed towards shared
regions common to both gag/pol and gag/prot.
          Implementation of this immunostimulant applica-
tion in humans requires that (1) the gene coding for the
antigen of interest be delivered to cells, (2) the antigen
25 be expressed in appropriate cells, and (3) MHC restriction
requirements, i.e., class I and class II antigen
interaction, are satisfied. Within a preferred
embodiment, preparations of vector are made by growing the
producer cells in normal medium, washing the cells with
30 PBS plus human serum albumin (HSA) at 10 mg/ml, then
growing the cells for 8-16 hours in PBS plus HSA. Titres
obtained are typically 10^4 to 10^6/ml depending on the
vector, packaging line or particular producer line clone.
The vector supernatants are filtered to remove cells and
35 are concentrated up to 100-fold by filtration through
100,000 or 300,000 pass Amicon filters (Wolff et al.,
Proc. Natl. Acad. Sci. 84:3344, 1987) or other equivalent

filters.   This lets globular proteins of 100,000 or
300,000 pass but retains 99% of the viral vector as
infectious particles.   The stocks can be frozen for
storage since they lose about 50% of the infectious units
5 on freezing and thawing.   Alternatively, the viral vector
can be further purified by conventional techniques.   The
most direct delivery involves administration of the
appropriate gene-carrying vector into the individual and
reliance upon the ability of the vector to efficiently
10 target to the appropriate cells, which can then initiate
stimulation of the immune response.   The dose is generally
10^5 to 10^6 infectious units/kg body weight.   The following
example demonstrates the ability of direct vector
injection to stimulate an immune response in mice.
15
B.   Stimulation Of An Immune Response In Mice By Direct
       Injection Of Retroviral Vector
              Experiments were performed to evaluate the
ability of recombinant retroviral vectors to induce
20 expression of HIV envelope proteins following direct
injection in mice.   Approximately 10^4 to 10^5 colony
forming units (cfu) of recombinant retrovirus carrying the
KT-1 vector construct were injected twice (2x) at 3-week
intervals either by the intraperitoneal (I.P.) or
25 intramuscular (I.M.) route.   This amount of retrovirus was
determined to be equivalent to approximately less than 100
μg of protein, which is usually considered too little to
stimulate an immune response.   Spleen cells were prepared
for CTL approximately 7 to 14 days after the second
30 injection of vector and CTL were restimulated in vitro
using irradiated BCenV stimulator cells as described above
(see Example 2A).   The results presented in Figure 4N
illustrate that direct vector injection by the I.P. and
I.M. routes stimulates the development of CTL which kill
35 BCenv target cells (I.M. 2x; I.P. 2x) but not control B/C
cells (B/C).   Thus, the injection of 10^4 to 10^5 units of
retrovirus (an amount of protein antigen which would not

usually stimulate an immune response) may induce
expression of significant levels of HIV envelope in the
autologous host cells which thereby leads to the induction
of a specific CTL immune response.

5   However, a more practical approach may involve
the extracorporeal treatment of patient peripheral blood
lymphocytes (PBL), fibroblasts or other cells obtained
from each individual with the vector, producer cells, or
vector plasmid DNA.   PBL can be maintained in culture
10 through the use of mitogens (phytohemagglutinin) or
lymphokines (e.g., IL-2).   The following example
demonstrates the ability of extracorporeal treatment of
primate cells to induce expression of vector encoded
proteins.

15

   C.   Extracorporeal Treatment Of Human Cells To Induce
        Expression Of Vector Encoded Proteins

         Experiments were conducted in which human
peripheral blood leukocytes (PBL) or fibroblasts and
20 chimpanzee dermal fibroblasts were infected with a murine
retroviral vector construct in which the cytomegalovirus
(cmv) promoter drives expression of β-galactosidase (cmvβ-
gal) as a marker enzyme for retroviral gene expression.
(A more detailed discussion of the use of such "Expression
25 Markers" appears in Section V, below; and engineering
retroviral vector constructs carrying the β-gal marker is
detailed also in Example 7, below).   Infection was
accomplished either by (a) electroporation (250V) or
transfection (CaPO$_4$/polybrene) of recombinant retrovirus
30 vector plasmid DNA, or (b) viral infection of cells by
recombinant retrovirus carrying vector construct RNA by
using the method of co-cultivating the cells with
irradiated retroviral vector producer cells, or (c) by
direct infection of cells by retroviral vector particles.
35 The results presented in Table I illustrates the infection
of human and primate cells by recombinant β-gal retroviral
vector constructs.   The results illustrate expression of

40

the β-galactosidase marker enzyme which can be visualized
by histochemical staining method, i.e., resulting in the
development of cells which carry a blue color (blue
cells).

5

Table I
EXTRA CORPOREAL INFECTION OF PRIMATE CELLS
WITH RETROVIRAL VECTOR CONSTRUCTS

CELLS	INFECTION/ CONDITION	VECTOR CONSTRUCT (AMOUNT)	RESULTS*
Human PBL			
PBL			
PBL	Electroporate/250V	cmvβgal(90μg)	÷,÷/-
PBL+PHA	Electroporate/250V	cmvβgal(90μg)	÷÷,÷
		cmvβgal(20μg)	+/-
PBL+PHA+IL2	Electroporate/250V	cmvβgal(90μg)	÷,÷
		cmvβgal(50μg)	+,÷/-
PBL#1÷PLB#2 (MLR)	Electroporate/250V	cmvβgal(90μg)	÷÷,÷/-
PBL#1÷PLB#2 (MLR)	Co-cultivation with Irradiated Producer	MLV/Neoβgal (10^2-10^4 pfu/ml)	÷÷,÷÷÷
Human Fibroblast			
AF-2	CaPO₄/polybrene Retroviral infection	cmvβgal(10μg) MLVβgal (10^5 pfu)	20-40% ÷÷÷
Vandenberg	CaPO₄/polybrene Retroviral infection	cmvβgal(10μg) MLVβgal (10^5 pfu)	10-15% ÷÷÷
Detroit 551	CaPO₄/polybrene Retroviral infection	cmvβgal(10μg) MLVβgal (10^5 pfu)	5% ÷÷÷
Chimpanzee Fibroblast			
X80	Retroviral infection	MLVβgal	÷÷

*Results of two experiments (separated by ","); a +/- indicates 20-99 blue cells; a ÷ indicated 100-499 blue cells; a ÷÷ indicates 500-1499 blue cells; and ÷÷÷ indicates greater than 1500 blue cells, out of a total of 2-3 X 10^9 total cells in the assay; % indicates that 5 to 40 percent of cells in the assay were infected as judged by the presence of blue cells after staining.

This type of approach allows for infection, monitoring of expression and expansion of the antigen presenting cell population prior to injection, and return of vector-expressing cells to the respective patient. Other types 5 of cells can also be explanted, vector introduced, and the cells returned to the patient. Only a moderate number of infected cells (10^5-10^7) is necessary to elicit strong immune responses in mice. It is probable that the dose to elicit an immune response is roughly the same per 10 individual animal or patient with very little dependence on body size.

Within one alternative method, cells are infected ex vivo as described above, and either inacti-vated by irradiation (see Figure 4E) or killed by fixa-15 tion, such as by formalin. Formalin fixation of cells treated with a vector expressing HIV env after treatment with the vector carrying the HIV env gene induces a strong CTL response.

Within another alternative method, stimulator 20 cell membrane fragments which contain both the antigen of interest and the appropriate MHC molecule as a complex are employed. Cells are infected with vector, genes expressed, cells disrupted and the membranes purified by centrifugation or affinity columns specific for the MHC-25 antigen complex. This process provides greater quality control from a manufacturing and stability standpoint.

Within yet another alternative method, an immune response is stimulated in tissue culture, instead of in the patient, and the immune cells are returned to the 30 patient. The following example demonstrates the ability of this type of approach to induce immune CTL in tissue culture.

D.   In Vitro Induction Of Immune Response To Retroviral
35   Vector-encoded Antigens

Experiments were conducted to evaluate the ability of retroviral vector encoded antigens to induce an

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in vitro immune response in human peripheral blood
leukocytes. PBL were prepared from donor #99 blood by
leukopheresis and Ficoll-Hypaque density gradient
sedimentation, and were stored frozen until use in liquid
5   nitrogen in RPMI medium containing 20% FBS and 10%
dimethylsulfoxide. To prepare EBV-transformed cell lines,
freshly thawed PBL from donor #99 were depleted of
T lymphocytes using OKT3 antibody and complement (or
cyclosporin treatment), and 1 ml of EBV-containing culture
10   supernatant from the B95 EBV-transformed lymphoblastoid
cell line was added for each 5×10^6 T-depleted PBL in a
total volume of 5 ml RPMI medium containing 2% human A-
minus serum. The EBV-infected cells were distributed into
96 well round bottom tissue culture microtiter plates
15   (200 μl per well) and placed in tissue culture at 37°C in
95% air/5% CO_2 until visible cell pellets were observed in
the bottom of the wells, after which time, the cells were
removed from the wells, expanded in tissue culture dishes
and flasks and periodically passaged. To prepare EBV-
20   HIVenv stimulator cells for _in vitro_ immunization, 10^7
EBV-transformed PBL were treated in 10 ml RPMI medium with
10^4 cfu/ml of tissue culture supernatant (or purified
virus) from a producer cell line productively-infected
with the KT-1 HIVenv retroviral vector. This vector also
25   contains the neomycin drug resistance gene which confers
resistance to G418 (a neomycin analogue). After overnight
incubation at 37°C in tissue culture the infected EBV-
transformed cells were collected by centrifugation and
resuspended in 1 ml of RPMI medium containing 10% FBS.
30   EBV-transformed HIVenv expressing cells were selected for
neomycin-resistance by adding 600 μg/ml of G418 to the
medium and distributing 100 μl into each well of a 96 well
round bottom microtiter plate. The plates were placed in
tissue culture at 37°C until visible cell pellets were
35   observed, at which time, the cells were removed, expanded,
and periodically passaged in tissue culture. HIVenv
expression was verified by Western blot analysis using

monoclonal or goat antibodies to HIV envelope proteins to
identify gp160 and gp120 on nitrocellulose blots of SDS-
PAGE gels. Alternatively, HIVenv expression was verified
by assaying for the ability of EBV-transformed HIVenv-
5 expressing cells to induce SupT1 cells to form
multicellular syncytia, i.e., a property of SupT1 cells
induced by some HIV envelope proteins. Finally, the donor
#99 EBV-transformed HIV-env-expressing lymphoblastoid
stimulator cells were irradiated at 10,000 R to inhibit
10 cell replication. To prepare in vitro immunized human
effector PBL, 10^7 freshly-thawed donor #99 cells were
mixed with 10^6 EBV-transformed HIV env-expressing
stimulator cells in RPMI 1640 containing 5% heat
inactivated A-minus human serum with pyruvate and non-
15 essential amino acids and the cell mixture was incubated
at 37°C for 7 days in tissue culture at a final density of
$1-5 \times 10^6$ cells per ml. After 7 days, the in vitro-
immunized effector cells were restimulated (in the same
manner as described above), for an additional 5 to 7 days
20 using a second addition of irradiated stimulator cells.
To prepare target cells for the CTL assays, irradiated
EBV-transformed HIV env stimulator cells were incubated
for 1 hour with ^{51}Cr, as described previously in
Example 2A. The ^{51}Cr release killing assay was also
25 performed as described previously (Example 2A, above) but
using donor #99 effector and target cells.

The results presented in Figure 40 illustrate in
vitro immunization of human donor #99 PBL to kill
autologus EBV-transformed lymphoblastoid target cells
30 expressing HIV envelope proteins (99-EBV-HIVenv) encoded
by the KT1 retroviral vector. The results also show
killing of autologous EBV-transformed cells (99-EBV)
resulting from a natural immunity to EBV (i.e., prior EBV
exposure as evidenced by the presence of antibody to EBV
35 in the serum from donor #99). In vitro immunized donor
#99 effector cells also showed a low level of killing of
normal autologous PHA-stimulated lymphocytes (PHA blasts)

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at the 100:1 effector to target cell ratio, perhaps
attributable to reactivation of latent EBV in these cells
during in vitro culture.

       This approach also allows the use of cells that
5   do not express human MHC molecules or express decreased
levels of MHC (e.g., human cell mutants, tumor cells,
mouse cells). Individual MHC class I or class II genes
are infected into MHC-cells to give expression of the
individual corresponding MHC protein, in a particular cell
10   line. The following example demonstrates the ability of
this type of approach to induce expression of MHC proteins
in a particular cell line which are functionally-active in
presenting antigen to immune CTL.

15   E.   Infection of Cells with both MHC and Antigen
       Recombinant infectious retrovirus carrying
murine H-2 genes are reported to induce expression and
presentation of H-2 antigens so that cells infected with
the retrovirus serve as target cells for lysis by
20   allogeneic immune murine CTL (Weis, J. H. et al., Molec.
Cell Biol. 5:1379-1384, 1985). In this case the H-2
antigen and its peptidic antigenic fragments are
synthesized by the target cell and become associated with
homologous "self" MHC molecules that are the natural
25   biosynthetic product of the target cell.

       It was reasoned that it may be possible to have
antigens presented by MHC molecules that are not the
natural biosynthetic product of the cell when (a) the MHC
gene is introduced into cells in a manner that permits
30   proper expression of the gene and synthesis, folding, and
processing of its protein product so that it is
functionally active in the cell, and (b) the antigen gene
is also introduced in a manner that would allow gene
expression with synthesis of the antigen protein and
35   appropriate association of its peptide antigenic fragments
with the MHC molecules. To evaluate infection of cells
with both antigen and MHC genes, and simultaneous

expression of both gene products, experiments were conducted using murine H2-Dd as the MHC gene, HIVenv as the antigen, human HT1080 cells as the target cells, and murine HIVenv immune CTL as the effector cells. In this
5   case the human cell must properly express the murine H2-Dd protein and the HIV envelope protein; the HIVenv peptidic antigen fragment must become associated with functional murine H2-Dd proteins; and, finally the H2-Dd peptidic HIVenv-H2-Dd complex in the human cell must properly
10 present the antigen in the complex to HIVenv-immune murine CTL for the human target cell to be killed. The results presented in Figure 4P illustrate that HT1080 cells transfected with murine H2-Dd and subsequently infected with retroviral vector carrying the KT-1 HIVenv vector
15 construct (HT1080 + Dd + env) present antigen and are killed by CTL that were induced in mice by immunization with murine BC10 env-29 cells, as described above (see Example 2A). These HIVenv-immune CTL kill in an antigen-specific manner, i.e., there was no lysis of control
20 HT1080 cells or BC cells expressing only H-2Dd (BC-Dd) and not HIVenv.

In a similar manner, tumor cells may be infected with an MHC gene and a tumor antigen in order to promote either the induction of immune CTL or the killing of the
25 tumor cells by immune CTL. In this manner autologous (i.e., from the patient), allogeneic (i.e., from another human donor), or xenogeneic (i.e., from an animal) CTL may be used to promote killing of human tumor cells by infecting the tumor cell with both an appropriate MHC
30 molecule and a tumor antigen.

A bank of cell lines capable of displaying antigens in the context of different MHC classes may also be generated by infecting cells with an MHC gene (or fragment thereof) or with both an MHC gene and an antigen
35 gene (or fragment thereof). A small number of these (10-20) will cover (i.e., have a match with) the majority of the human population. For example, HLA A2 is present in

about 30-60% of individuals. In the case of non-human
cells, those can be derived from transgenic animals (such
as mice) which express human MHC molecules generally or in
specific tissues due to the presence of a transgene in the
5   strain of animals (see, e.g., Chamberlin et al., Proc.
Natl. Acad. Sci. USA 85:7690-7694, 1988). These cell
lines may be useful for mapping the peptidic antigenic
fragments in tumor cell antigens or infectious agents
which represent the major immunodominant epitopes of the
10 protein for CTL or antibody induction.

In any of the above situations, the presentation
or response to the presentation can be enhanced by also
infecting into the cells genes of other proteins involved
in the immune interactions which are missing or
15 underrepresented (e.g., β microglobulin, LFA3, CD3, ICAM-I
and others). β microglobulin is a nonvariant, necessary
subunit of the class I MHC, CD3 is involved in the MHC
interaction, and LFA3 and ICAM-I molecules enhance the
interaction of cells of the immune system (see, e.g.,
20 Altmann et al., Nature 338:512, 1989) leading to stronger
responses to the same level of immune stimulation.

In the case of transgenic mice expressing human
MHC, the stimulation could also be performed in the mouse
using somatic transgenic mouse cells expressing a foreign
25 antigen, the gene for which was introduced by a viral
vector or other means, as stimulators. The mouse CTL thus
generated would have T-cell receptors expressing in the
context of the human MHC, and could be used for passive
cellular immunization or treatment (i.e., infused into
30 patients) of patients.

As a further alternative, one can use cells from
a patient and boost expression of "self" MHC class I genes
by introducing the matched MHC gene by vector transfer or
other means including the use of genes encoding proteins
35 (e.g., interferons) which stimulate MHC expression or the
use of regulatory elements which control expression of MHC
gene expression. Such a boost in MHC I expression causes

more efficient presentation of foreign antigens, whether
they are present already in the patient's cells (e.g.,
tumor cells) or subsequently added using viral vectors
encoding foreign antigens. This, in turn, leads to a more
5 potent immune response when even cells with reduced MHC I
expression (such as some virally infected cells or some
tumor types) are efficiently eliminated. Within certain
aspects of the present invention, one can infect
susceptible target cells with a combination or permutation
10 of nucleic acid sequences encoding (a) individual Class I
or Class II MHC protein, or combinations thereof;
(b) specific antigens or modified forms thereof capable of
stimulating an immune response; (c) both MHC and
antigen(s); and (d) other proteins involved in the immune
15 interactions which are missing or underrepresented, as
discussed above. The respective steps of infection may be
performed _in vivo_ or _ex vivo_. The immune CTL induction
may be performed either _ex vivo_ or _in vivo_ and the killing
of the specific cell types may be effected _ex vivo_ or
20 _in vivo_.

A different form of administration is the
implantation of producer lines making retroviral vector
particles. These may be immunologically unmatched
classical producer cell lines or the patients own cells,
25 which have been explanted, treated and returned (see VI
Alternative Viral Vector Packaging Techniques, below).
Both types of implants (10^5 - 10^6 cells/kg body weight)
would have a limited life span in the patient, but would
lead to the retroviral vector infecting large numbers (10^7
30 - 10^{10}) of cells in their vicinity in the body.

In any case, the success of the HIV immune
stimulating treatment can be assayed by removing a small
amount of blood and measuring the CTL response using as
targets the individual's own cells infected with vector
35 leading to env expression.

When it is desired to stimulate an MHC class I
or class II restricted immune response to pathogens,

including pathogenic viruses other than HIV, suitable
forms of envelope or other antigens associated with such
retroviruses which will stimulate an immune response can
be ascertained by those skilled in the art. In general,
there will be combinations of epitopes which cause induc-
tion of various parts of the immune system (e.g., T_H-,
T_C-, B-cells).   In addition, some epitopes may be
pathogenic or hypervariable but immunodominant.   The
present invention allows a "mix-and-match" selection of
combinations of desirable epitopes and exclusion of
undesirable epitopes. For example, in HIV, a number of
hypervariable loops which carry immunodominant B- and
T-cell epitopes can be strung together in the gene
sequence carried by the vector so that the resultant
immunostimulation is appropriate for the preponderance of
HIV strains found clinically.   The following example
illustrates procedures using retroviral vectors for
identifying and mapping CTL-modified forms thereof.

F.   <u>Vectors   Expressing   HIV   Antigenic   Epitopes   and
      Modified   Forms   Thereof   for   Mapping   the   Immune
      Response</u>

        The results presented above in Example 2 (above)
illustrate that mice immunized with retroviral vector-
encoded HIVenv develop significant class I MHC-restricted
CTL responses that are specific for HIVenv. Further, the
results presented in Example 2 illustrate that HIVenvIIIB
induced CTL which exhibit lytic activity on target cells
coated with synthetic peptides derived from the gp120
hypervariable region of both HIV-IIIB and HIV-MN variants.
To further map the regions within the HIV envelope gene
encoding peptidic antigen fragments reactive in inducing
and stimulating CTL, and in mediating lysis of targets by
immune CTL, experiments were conducted with the "Δ loop"
and "Churx I" recombinant retroviral vector constructs
described in Example 1F, above. The "Δ loop" vector is an
HIV-IIIB env-encoding vector construct which has a
deletion of the gp120 hypervariable loop in the V3 domain

of the envelope gene so that a truncated protein may be produced containing only the non-loop ("loopless") portions of HIVenv.

5   To map peptidic antigenic epitopes within the HIV envelope protein reactive with CTL, experiments were conducted in which retrovirus carrying the "Δ loop" vector construct was used to infect BC cells and thereby create cells expressing envelope proteins having the truncated form of "loopless" envelope, i.e., lacking the gp120 10 hypervariable loop (BCenvΔV3 cells). These "loopless" HIVenv cells were tested for their ability to induce HIVenv-immune CTL in mice. The results presented in Figure 4A include control assays, in panel A, and experimental assays in panel B. The results illustrate 15 that CTL are induced in mice by "loopless" BCenvΔV3 cells and these CTL kill both BCenvΔV3 cells (panel 1B, Figure 4Q) and BCenvΔV3 target cells expressing a full-length envelope protein. Specificity of the CTL was illustrated by their failure to lyse BC cells (i.e., not expressing 20 HIVenv) or BC cells coated with the RP135 synthetic "loop" peptide (BC-RP135), i.e., lacking in the BCenvΔV3 cells used to induce the CTL. The results of control assays presented in Figure 4Q, panel A, illustrate that BC cells coated with RP135 peptide (BC-RP135) effectively-presented 25 antigen to BCenv-"loop"-immune CTL (i.e., BCenv-immune CTL) and were killed by these effector cells in a specific manner, i.e., the BCenv-immune CTL did not kill BC cells (the negative control) but did kill BCenv target cells (the positive control). Thus, retrovirus-carrying 30 recombinant vector constructs can be used to identify and map regions of the HIV envelope protein that induce CTL and confer lysability upon target cells.

To further map the peptidic antigenic regions within the HIV envelope, experiments were conducted in 35 which the "Chunk 1" vector construct was used to infect BC cells and thereby create cells expressing a "loopless" envelope protein which also lacks peptide sequences in

regions adjacent to the gp120 loop. The results presented
in panel A of Figure 4R illustrate that target cells
expressing "Chunk 1" (i.e., BCpCMV Chunk1 target) are
lysed in a specific manner by HIVenv-immune CTL, i.e.,
5   these CTL do not lyse BC10ME cells (the negative control)
but do lyse BC cells expressing HIVenv (BCenvIIIB target).
The results presented in panel B of Figure 4R also
illustrate that BCpCMV Chunk 1 target cells and BC cells
expressing HIVenv (BCenv IIIB 14H target) are lysed but
10 not BC10ME cells (BE10ME target).

An alternative approach to creating a desired
immune response is to deliver an antigen-specific T-cell
receptor gene to an appropriate cell, such as a T-cell.
It is also possible to molecularly graft the genetic
15 message for antigen recognition sites of immunoglobulin
molecules into the corresponding sites in the genes of the
related T-cell receptor subunits α and β. Such altered
protein molecules will not be MHC restricted, and will be
able to perform as T_H- and T_C-cells specific for the
20 antigen defined by the original immunoglobulin. Another
tactic is to transfer genes for effector molecules in NK
into NK cells to confer additional non-MHC limited killing
capability on these cells. In addition, specific
immunoglobulin genes could similarly be useful when
25 delivered to B-cells to cause the large-scale in vivo
production of a particular antibody molecule in a patient.

II. Blocking Agents

Many infectious diseases, cancers, autoimmune
30 diseases, and other diseases involve the interaction of
viral particles with cells, cells with cells, or cells
with factors. In viral infections, viruses commonly enter
cells via receptors on the surface of susceptible cells.
In cancers, cells may respond inappropriately or not at
35 all to signals from other cells or factors. In autoimmune
disease, there is inappropriate recognition of "self"
markers. Within the present invention, such interactions

may be blocked by producing, <u>in vivo</u>, an analogue to either of the partners in an interaction.

This blocking action may occur intracellularly, on the cell membrane, or extracellularly. The blocking
5 action of a viral or, in particular, a retroviral vector carrying a gene for a blocking agent, can be mediated either from inside a susceptible cell or by secreting a version of the blocking protein to locally block the pathogenic interaction.

10   In the case of HIV, the two agents of inter-action are the gp 120/gp 41 envelope protein and the CD4 receptor molecule. Thus, an appropriate blocker would be a vector construct expressing either an HIV env analogue that blocks HIV entry without causing pathogenic effects,
15 or a CD4 receptor analogue. The CD4 analogue would be secreted and would function to protect neighboring cells, while the gp 120/gp 41 is secreted or produced only intracellularly so as to protect only the vector-containing cell. It may be advantageous to add human
20 immunoglobulin heavy chains or other components to CD4 in order to enhance stability or complement lysis. Delivery of a retroviral vector encoding such a hybrid-soluble CD4 to a host results in a continuous supply of a stable hybrid molecule.

25   Vector particles leading to expression of HIV env may also be constructed as described above. It will be evident to one skilled in the art which portions are capable of blocking virus adsorption without overt pathogenic side effects (Willey et al., <u>J. Virol.</u> <u>62</u>:139,
30 1988; Fisher et al., <u>Science</u> <u>233</u>:655, 1986). The follow-ing example describes the construction of a CD4 vector from which infectious vector particles were made (Figure 5).

EXAMPLE 3

sCD4 Vector

1.   A 1.7 kb Eco R1 - Hind III DNA fragment
from pMV7.T4 (Maddon et al., Cell 47:333, 1986) was blunt-
5   end ligated to the Hinc II site of Sk⁺.

2.   A universal translation termination
sequence containing an Xba I site was inserted into the
Nhe I site of the CD4 fragment.

3.   The 1.7 kb Xho I - Cla I fragment was
10   excised and cloned into the Xho I - Cla I site of pAFVXM.
These vector plasmids can be used to generate infectious
vector particles, as described in Example 1.

Such infectious blocking vectors, when put into
human T-cell lines in culture, can inhibit the spread of
15   HIV infections. Preparation, concentration and storage of
infectious retroviral vector preparations is as for the
immunostimulant. Route of administration would also be
the same, with doses about tenfold higher. Another route
which may be used is the aspiration of bone marrow,
20   infection with retroviral vector and return of this
infected marrow (Gruber et al., Science 230:1057, 1985) to
the patient. Since the marrow replication will amplify
the vector expression through cell replication, doses in
the range of the immunostimulant can be used ($10^5 - 10^6$/kg
25   body weight).

In any case, the efficacy of the treatment can
be assayed by measuring the usual indicators of disease
progression, including antibody level, viral antigen
production, infectious HIV levels, or levels of
30   nonspecific infections.

III. Expression of Palliatives

Techniques similar to those described above can
be used to produce recombinant retroviruses with vector
35   constructs which direct the expression of an agent (or
"palliative") which is capable of inhibiting a function of
a pathogenic agent or gene. Within the present invention,

"capable of inhibiting a function" means that the palliative either directly inhibits the function or indirectly does so, for example, by converting an agent present in the cells from one which would not normally
5   inhibit a function of the pathogenic agent to one which does. Examples of such functions for viral diseases include adsorption, replication, gene expression, assembly, and exit of the virus from infected cells. Examples of such functions for a cancerous cell or
10 cancer-promoting growth factor include viability, cell replication, altered susceptibility to external signals (e.g., contact inhibition), and lack of production or production of mutated forms of anti-oncogene proteins.

15   (i)   Inhibitor Palliatives
              In one aspect of the present invention, the recombinant retrovirus carries a vector construct which directs the expression of a gene which can interfere with a function of a pathogenic agent, for instance in viral or
20 malignant diseases.   Such expression may either be essentially continuous or in response to the presence in the cell of another agent associated either with the pathogenic condition or with a specific cell type (an "identifying agent").   In addition, vector delivery may be
25 controlled by targeting vector entry specifically to the desired cell type (for instance, a virally infected or malignant cell) as discussed below.
              A preferred method of administration is leukophoresis, in which about 20% of an individual's PBLs
30 are removed at any one time and manipulated in vitro. Thus, approximately 2×10^9 cells may be treated and replaced.   Since the current maximum titres are around 10^6/ml, this requires 2 to 20 liters of starting viral supernatant.   Repeat treatments also would be performed.
35 Alternatively, bone marrow may be treated and allowed to amplify the effect as described above.

In addition, packaging cell lines producing a
vector may be directly injected into a subject, allowing
continuous production of recombinant virions. Examples of
suitable cell types include monocytes, neutrophils, or
5   their progenitors, since these cells are present in the
peripheral blood but can also leave the circulatory system
to allow virus production in extravascular tissue
(particularly the central nervous system) where virion
production may be therapeutically required. Such a cell
10 line would ultimately be rejected as foreign by the host
immune system. To ensure the eventual destruction of
these foreign cells from the host (even an immuno-
suppressed host) the cell line may be engineered to
express the gene for a conditionally lethal protein, such
15 as HSVTK. Thus, administration of the drug Acyclovir
(ACV) (a drug which is specifically toxic for cells
expressing HSVTK) eliminates these cells after sufficient
vector has been produced _in vivo_. Such a packaging cell
line could be a continuous cell line or could be made
20 directly from host cells.

In one embodiment, retroviral viruses which
express RNA complementary to key pathogenic gene
transcripts (for example, a viral gene product or an
activated cellular oncogene) can be used to inhibit
25 translation of that transcript into protein, such as the
inhibition of translation of the HIV tat protein. Since
expression of this protein is essential for viral repli-
cation, cells containing the vector would be resistant to
HIV replication. To test this, the vector αtat (Figure
30 10) has been constructed, packaged as recombinant virions
and introduced into human T-cells and monocyte cell lines
in the absence of replication-competent helper virus.

In a second embodiment, where the pathogenic
agent is a single-stranded virus having a packaging
35 signal, RNA complementary to the viral packaging signal
(e.g., an HIV packaging signal when the palliative is
directed against HIV) is expressed, so that the associa-

tion of these molecules with the viral packaging signal will, in the case of retroviruses, inhibit stem loop formation or tRNA primer binding required for proper encapsidation or replication of the retroviral RNA genome.

In a third embodiment, a retroviral vector may be introduced which expresses a palliative capable of selectively inhibiting the expression of a pathogenic gene, or a palliative capable of inhibiting the activity of a protein produced by the pathogenic agent. In the case of HIV, one example is a mutant tat protein which lacks the ability to transactivate expression from the HIV LTR and interferes (in a transdominant manner) with the normal functioning of tat protein. Such a mutant has been identified for HTLV II tat protein ("XII Leu^5" mutant; see Wachsman et al., Science 235:674, 1987). A mutant transrepressor tat should inhibit replication much as has been shown for an analogous mutant repressor in HSV-1 (Friedmann et al., Nature 335:452, 1988).

Such a transcriptional repressor protein may be selected for in tissue culture using any viral-specific transcriptional promoter whose expression is stimulated by a virus-specific transactivating protein (as described above). In the specific case of HIV, a cell line expressing HIV tat protein and the HSVTK gene driven by the HIV promoter will die in the presence of ACV. However, if a series of mutated tat genes are introduced to the system, a mutant with the appropriate properties (i.e., represses transcription from the HIV promoter in the presence of wild-type tat) will grow and be selected. The mutant gene can then be reisolated from these cells. A cell line containing multiple copies of the condition- ally lethal vector/tat system may be used to assure that surviving cell clones are not caused by endogenous muta- tions in these genes. A battery of randomly mutagenized tat genes are then introduced into these cells using a "rescuable" retroviral vector (i.e., one that expresses the mutant tat protein and contains a bacterial origin of

replication and drug resistance marker for growth and selection in bacteria). This allows a large number of random mutations to be evaluated and permits facile subsequent molecular cloning of the desired mutant cell line.
5   This procedure may be used to identify and utilize mutations in a variety of viral transcriptional activator/viral promoter systems for potential antiviral therapies.

        In a fourth embodiment, the recombinant retro-
10   virus carries a vector construct that directs the expression of a gene product capable of activating an otherwise inactive precursor into an active inhibitor of the pathogenic agent. For example, the HSVTK gene product may be used to more effectively metabolize potentially
15   antiviral nucleoside analogues, such as AZT or ddC. The HSVTK gene may be expressed under the control of a constitutive macrophage or T-cell-specific promoter and introduced into these cell types. AZT (and other nucleoside antivirals) must be metabolized by cellular
20   mechanisms to the nucleotide triphosphate form in order to specifically inhibit retroviral reverse transcriptase and thus HIV replication (Furmam et al., _Proc. Natl. Acad. Sci. USA_ 33:8333-8337, 1986). Constitutive expression of HSVTK (a nucleoside and nucleoside kinase with very broad
25   substrate specificity) results in more effective metabolism of these drugs to their biologically active nucleotide triphosphate form. AZT or ddC therapy will thereby be more effective, allowing lower doses, less generalized toxicity, and higher potency against
30   productive infection. Additional nucleoside analogues whose nucleotide triphosphate forms show selectivity for retroviral reverse transcriptase but, as a result of the substrate specificity of cellular nucleoside and nucleotide kinases are not phosphorylated, will be made
35   more efficacious. A description of a representative method is set forth in Example 4.

EXAMPLE 4
Vectors Designed to Potentiate the Antiviral Effect
of AZT and Analogues

A.   All of the following retroviral vectors are
based on the "N2" vector (see Keller et al., Nature
318:149-154, 1985). Consequently, 5' and 3' Eco R1 LTR
fragments (2.8 and 1.0 kb, respectively) were initially
subcloned into plasmids containing polylinkers (into SK+
to give pN2R5^{+/-}; into pUC31 to give p31N2R5^{+/-} and
p31N2R3^{+/-} to facilitate vector construction. pUC31 is
a modification of pUC19 carrying additional restriction
sites (Xho I, Bgl II, BssH II, and Nco I) between the
Eco R1 and Sac I sites of the polylinker. In one case, a
1.2 kb Cla I/Eco R1 5' LTR fragment was subcloned into the
same sites of an SK+ vector to give pN2CR5. In another
case, the 5' LTR containing a 6 bp deletion of the splice
donor sequence was subcloned as a 1.8 kb Eco R1 fragment
into pUC31 (p31N25delta[+]). The coding region and
transcriptional termination signals of HSV-1 thymidine
kinase gene were isolated as a 1.8 kb Bgl II/Pvu II
fragment from plasmid 322TK (3.5 kb Bam HI fragment of
HSVTK cloned into Bam HI of pBR322) and cloned into
Bgl II/Sma I-digested pUC31 (pUCTK). For constructs which
require deletion of the terminator signals, pUCTK was
digested with Sma I and Bam HI. The remaining coding
sequences and sticky-end Bam HI overhang were
reconstituted with a double-stranded oligonucleotide made
from the following oligomers:

          5' GAG AGA TGG GGG AGG CTA ACT GAG 3'

     and 5' GAT CCT CAG TTA GCC TCC CCC ATC TCT C 3'
forming the construct pTK delta A.

          For diagnostic purposes, the oligos were
designed to destroy the Sma I site while keeping its Ava I
site without changing the translated protein.

          The 0.6 kb HIV promoter sequences were cloned as
a Dra I/Hind III fragment from pCV-1 (see Arya et al.,

Science 229:69-73, 1985) into Hinc II/Hind III-cut SK⁻
(SKHL).

B.   Construction of TK-1 and TK-3 retroviral vectors (see
5   Figure 6).

1.   The 5 kb Xho I/Hind III 5′ LTR and plasmid
sequences were isolated from p31N2R5(+).

2.   HSVTK coding sequences lacking transcrip-
tional termination sequences were isolated as a 1.2 kb
10 Xho I/Bam HI fragment from pTKdeltaA.

3.   3′ LTR sequences were isolated as a 1.0 kb
Bam HI/Hind III fragment from pN2R3(-).

4.   The fragments from steps 1-3 were mixed,
ligated, transformed into bacteria, and individual clones
15 identified by restriction enzyme analysis (TK-1).

5.   TK-3 was constructed by linearizing TK-1
with Bam HI, filling in the 5′ overhang and blunt-end
ligating a 5′-filled Cla I fragment containing the
bacterial lac UV5 promoter, SV40 early promoter, plus Tn5
20 Neo^r gene. Kanamycin-resistant clones were isolated and
individual clones were screened for the proper orientation
by restriction enzyme analysis.

These constructs were used to generate
infectious recombinant vector particles in conjunction
25 with a packaging cell line, such as PA317, as described
above.

Administration of these retroviral vectors to
human T-cell and macrophage/monocyte cell lines can
increase their resistance to HIV in the presence of AZT
30 and ddC compared to the same cells without retroviral
vector treatment. Treatment with AZT would be at lower
than normal levels to avoid toxic side effects, but still
efficiently inhibit the spread of HIV. The course of
treatment would be as described for the blocker.

35   Preparation, concentration and storage of the
retroviral vector preparations would be as described
above. Treatment would be as previously described but ex

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corpore treatment of patients' cells would aim for uninfected potentially susceptible T-cells or monocytes. One preferred method of targeting the susceptible cell is with vectors which carry HIV env or hybrid env (see
5   Section VIII Cell Line Specific Retroviruses, below) to direct absorption of vector particles to CD4$^+$ cells. Normal adults have about 5×10^9 T4 cells in their total blood and about the same number of monocytes.

A fifth embodiment for producing inhibitor
10 palliatives involves the delivery and expression of defective interfering viral structural proteins, which inhibit viral assembly. Vectors would code for defective gag, pol, env or other viral particle proteins or peptides, and these would inhibit in a dominant fashion
15 the assembly of viral particles. This occurs because the interaction of normal subunits of the viral particle is disturbed by interaction with the defective subunits.

A sixth such embodiment involves the expression of inhibiting peptides or proteins specific for viral
20 protease. Viral protease cleaves the viral gag and gag/pol proteins into a number of smaller peptides. Failure of this cleavage in all cases leads to complete inhibition of production of infectious retroviral particles. The HIV protease is known to be an aspartyl
25 protease, and these are known to be inhibited by peptides made from amino acids from protein or analogues. Vectors to inhibit HIV will express one or multiple fused copies of such peptide inhibitors.

A seventh embodiment involves the delivery of
30 suppressor genes which, when deleted, mutated or not expressed in a cell type, lead to tumorigenesis in that cell type. Reintroduction of the deleted gene by means of a viral vector leads to regression of the tumor phenotype in these cells. Examples of such cancers are
35 retinoblastoma and Wilms Tumor. Since malignancy can be considered to be an inhibition of cellular terminal differentiation compared with cell growth, the retroviral

delivery and expression of gene products which lead to differentiation of a tumor should also, in general, lead to regression.

In an eighth embodiment, the retroviral 5 construct (with or without the expression of a palliative) provides a therapeutic effect by inserting itself into a virus, oncogene, or pathogenic gene, thereby inhibiting a function required for pathogenesis. This embodiment requires the direction of retroviral integration to a 10 specific site in the genome by homologous recombination, integrase modification, or other methods (described below).

In an ninth embodiment, the retroviral vector provides a therapeutic effect by encoding a ribozyme (an 15 RNA enzyme) (Haseloff and Gerlach, Nature 334:585, 1989) which will cleave and hence inactivate RNA molecules corresponding to a pathogenic function. Since ribozymes function by recognizing a specific sequence in the target RNA and this sequence is normally 12 to 17 bp, this allows 20 specific recognition of a particular RNA species such as a RNA or a retroviral genome. Additional specificity may be achieved in some cases by making this a conditional toxic palliative (see below).

One way of increasing the effectiveness of 25 inhibitory palliatives is to express viral inhibitory genes in conjunction with the expression of genes which increase the probability of infection of the resistant cell by the virus in question. The result is a nonproductive "dead-end" event which would compete for 30 productive infection events. In the specific case of HIV, vectors may be delivered which inhibit HIV replication (by expressing anti-sense tat, etc., as described above) and also overexpress proteins required for infection, such as CD4. In this way, a relatively small number of vector-35 infected HIV-resistant cells act as a "sink" or "magnet" for multiple nonproductive fusion events with free virus or virally infected cells.

(ii) Conditional Toxic Palliatives

Another approach for inhibiting a pathogenic agent is to express a palliative which is toxic for the
5   cell expressing the pathogenic condition. In this case, expression of the palliative from the proviral vector should be limited by the presence of an entity associated with the pathogenic agent, such as an intracellular signal identifying the pathogenic state in order to avoid
10 destruction of nonpathogenic cells. This cell-type specificity may also be conferred at the level of infection by targeting recombinant retrovirus carrying the vector to cells having or being susceptible to the pathogenic condition.

15   In one embodiment of this method, a recombinant retrovirus (preferably, but not necessarily, a recombinant MLV retrovirus) carries a vector construct containing a cytotoxic gene (such as ricin) expressed from an event-specific promoter, such as a cell cycle-dependent promoter
20 (e.g., human cellular thymidine kinase or transferrin receptor promoters), which will be transcriptionally active only in rapidly proliferating cells, such as tumors. In this manner, rapidly replicating cells, which contain factors capable of activating transcription from
25 these promoters, are preferentially destroyed by the cytotoxic agent produced by the proviral construct.

In a second embodiment, the gene producing the cytotoxic agent is under control of a tissue-specific promoter, where the tissue specificity corresponds to the
30 origin of the tumor. Since the viral vector preferentially integrates into the genome of replicating cells (for example, normal liver cells are not replicating, while those of a hepatocarcinoma are), these two levels of specificity (viral integration/replication and tissue-
35 specific transcriptional regulation) lead to preferential killing of tumor cells. Additionally, event-specific and tissue-specific promoter elements may be artificially

combined such that the cytotoxic gene product is expressed
only in cell types satisfying both criteria (e.g., in the
example above, combined promoter elements are functional
only in rapidly dividing liver cells). Transcriptional
5 control elements may also be amplified to increase the
stringency of cell-type specificity.

These transcriptional promoter/enhancer elements
need not necessarily be present as an internal promoter
(lying between the viral LTRs) but may be added to or
10 replace the transcriptional control elements in the viral
LTRs which are themselves transcriptional promoters, such
that condition-specific transcriptional expression will
occur directly from the modified viral LTR. In this case,
either the condition for maximal expression will need to
15 be mimicked in retroviral packaging cell lines (e.g., by
altering   growth   conditions,   supplying   necessary
transregulators of expression or using the appropriate
cell line as a parent for a packaging line), or the LTR
modification is limited to the 3' LTR U3 region, to obtain
20 maximal recombinant viral titres. In the latter case,
after one round of infection/integration, the 3' LTR U3 is
now also the 5' LTR U3, giving the desired tissue-specific
expression.

In a third embodiment, the proviral vector
25 construct is similarly activated but expresses a protein
which is not itself cytotoxic, and which processes within
the target cells a compound or a drug with little or no
cytotoxicity into one which is cytotoxic (a "conditionally
lethal" gene product). Specifically, the proviral vector
30 construct carries the herpes simplex virus thymidine
kinase ("HSVTK") gene downstream and under the
transcriptional control of an HIV promoter (which is known
to be transcriptionally silent except when activated by
HIV tat protein). Expression of the tat gene product in
35 human cells infected with HIV and carrying the proviral
vector construct causes increased production of HSVTK.
The cells (either _in vitro_ or _in vivo_) are then exposed to

a drug such as acyclovir or its analogues (FIAU, FIAC, DHPG). These drugs are known to be phosphorylated by HSVTK (but not by cellular thymidine kinase) to their corresponding active nucleotide triphosphate forms (see, for example, Schaeffer et al., Nature 272:583, 1978). Acyclovir and FIAU triphosphates inhibit cellular polymerases in general, leading to the specific destruction of cells expressing HSVTK in transgenic mice (see Borrelli et al., Proc. Natl. Acad. Sci. USA 85:7572, 1988). Those cells containing the recombinant vector and expressing HIV tat protein are selectively killed by the presence of a specific dose of these drugs. In addition, an extra level of specificity is achieved by including in the vector the HIV rev protein, responsive CRS/CAR sequences. In the presence of the CRS sequence gene expression is suppressed, except in the presence of the CAR sequences and the rev protein. Example 5 provides an illustration of this technique.

EXAMPLE 5
Vector to Conditionally Potentiate the Toxic Action of ACV or Its Analogues
Construction of Vectors

    A.   Construction of pKTVIHAX (see Figure 7).

    1.   The 9.2 kb Asu II/Xho I fragment was isolated from vector pN2 DNA.

    2.   The 0.6 kb Xho I/Bam HI promoter fragment was isolated from plasmid pSKHL.

    3.   The 0.3 kb Bgl II/Acc I and 1.5 kb Acc I/Acc I fragment were purified from pUCTK.

    4.   The fragments from 1, 2, and 3 were ligated, transformed into bacteria, and appropriate Amp^r clones of the given structure identified by restriction enzyme analysis.

B.   Construction   of   pKTVIH-5   and   pKTVIH5   Neo
     retroviral vectors (see Figure 8).

          1.   The 4.5 kb 5' LTR and vector fragment was
isolated   as   an   Xho I/Bam   HI   fragment   from   vector
p31N25delta(+).

          2.   The 1.0 kb 3' LTR was isolated as an
Apa I/Bam HI fragment from pN2R3(+) fragment.

          3.   The 0.6 kb HIV promoter element was
isolated from pSKHL as an Apa I/Eco R1 fragment.

          4.   The   HSVTK   coding   sequence   and
transcriptional termination sequences were isolated as 1.8
kb Eco R1/Sal I fragment from pUCTK.

          5.   The fragments from 1-4 were combined,
ligated, transformed into bacteria, and clones of the
given structure were identified by restriction enzyme
analysis (pKTVIH-5).

          6.   Plasmid pKTVIH5 Neo was constructed by
linearizing pKTVIH5 with Cla I; mixing with a 1.8 kb Cla I
fragment containing the bacterial lac UV5 promoter, SV40
early   promoter,   and   Tn5   Neo^r marker,   ligating,
transforming bacteria and selecting for kanamycin
resistance.   Clones with the insert in the indicated
orientation were identified by restriction analysis.

C.   Construction of MHMTK Neo retroviral vector (see
     Figure 9).

          1.   Construction of intermediate plasmid MHM-1
LTR.

          a)   Plasmid pN2CR5 was linearized by
partial digestion with Fok I, the 5' overhang filled
in with deoxynucleotide triphosphates using Klenow
DNA polymerase, and Hind III linkers inserted. After
transformation into bacteria, a clone with a Hind III
linker inserted in the MLV LTR Fok I site was
identified by restriction enzyme analysis (pN2CR5FH).

          b)   Plasmid pN2CR5FH was linearized with
Nhe I, the 5' overhang filled in with Klenow

polymerase digested with Hind III, and the 4.3 kb fragment with promoterless MLV sequences isolated.

    c) 0.5 kb Eco RV/Hind III HIV promoter sequences were isolated from pSKHL.

    d) b and c were mixed, ligated, used to transform bacteria, and the structure of MHM-1 was confirmed by restriction enzyme analysis.

    2. The 0.7 kb Eco RV/Bal I fragment isolated from MHM-1 was subcloned into the Eco RV site of plasmid I30B (a modified IBI30 plasmid containing additional Bgl II, Bst II, Neo I and Nde I sites in the polylinker). After transformation into bacteria, clones with the appropriate orientation were identified by restriction enzyme analysis (pMHMB).

    3. Plasmid pMHMB was digested with Apa I and Xho I and gel purified.

    4. MHM-1 was digested with Apa I/Bam HI and the 1.8 kb MHMLTR/leader sequence gel purified.

    5. The 2.8 kb Bgl II/Sal I fragment containing the HSVTK coding region upstream of the SV40 early promoter driving Neo^r taken from pTK-3 (see Figure 3).

    6. 3-5 were mixed, ligated, used to transform bacteria, and appropriate clones were identified by restriction enzyme analysis.

    This vector and similar vectors which contain inducible elements in their LTR's result in an added safety feature. Briefly, since the LTR is inactive in the absence of HIV, insertional downstream activation of undesirable host genes (such as proto-oncogenes) does not occur. However, tat expression in the packaging cell line allows facile manipulation of the virion in tissue culture.

    D. **Construction of RRKTVIH retroviral vector (see Figure 10)**

    1. The 9.2 kb Asu II/Xho I fragment was isolated from vector pN2 DNA.

2.   The 0.6 kb Xho I/Eco R1 HIV promoter fragment was isolated from plasmid pSKHL.

3.   The HIV rev responsive HSVTK (RRTK) was constructed in the following manner:

a)   The HSVTK gene was subcloned as a 1.8 kb HinC II/Pvu II fragment into the Eco RV site of vector SK+ (pSTK[-]).

b)   The 1.8 kb Kpn I/Hind III fragment which contains the CRS/CAR elements from HIV env was repaired and blunt-end ligated into the Sma I site of vector I30B (pCRS/CAR^{+/-}). I30B is a modified IBI30 plasmid containing the same additional restriction sites as for pUC31 with an Nde I site instead of the IBI30 Xho I site.

c)   The 3.6 kb BssH II/Eco R1 fragment containing vector and HSVTK polyadenylation signals was isolated from pSTK(-),

d)   The 1.8 kb Bam HI/BssH II CRS/CAR fragment was isolated from pCRS/CAR(-).

e)   The 1.2 Eco R1/Bam HI coding sequence fragment was isolated from pTKdeltaA.

f)   C, D and E were ligated and appropriate recombinants screened by restriction enzyme analysis.

4.   Rev-responsive HSVTK was isolated as a 3.6 kb Eco R1/Cla I fragment.

5.   1, 2 and 4 were ligated and appropriate recombinants identified by restriction enzyme analysis.

E.   Construction of tat and anti-tat expression vectors (see Figure 11).

These vectors are used as pseudo-HIV to test-activate tat-dependent HSVTK vectors.

1.   The His^r expression vector pBamHis was linearized with Bam HI and treated with calf intestinal phosphatase.

2.   The Sac I site of pCV-1 was mutagenized to
a Bam HI site and the 350 bp Bam HI coding sequence of HIV
tat was isolated.

3.   The fragments purified in steps 1 and 2
5   were mixed, ligated, used to transform bacteria, and
clones with tat in both orientations (expressing tat or
the "anti-sense" tat) were identified by restriction
enzyme analysis.

These constructs were used to generate
10 infectious recombinant vector particles in conjunction
with a packaging cell line such as PA317, as described
above. These vectors are genetically stable and result in
predictable proviral structure as judged by Southern blot
analysis of restriction-enzyme-digested genomic DNA from
15 individual clones of infected cells (39/40 clones tested
had proviruses of the expected size).

The biological properties of these retroviral
vectors are described hereinafter. The HIV tat gene
("tathis" vector -- see Figure 11) was transfected into
20 mouse PA317 cells. Five individual histidinol-resistant
subclones were obtained (TH 1-5) which express HIV tat.
These cells are thus an experimental model for HIV infec-
tion. The vectors KTVIHAX, KTVIH5NEO, and MHMTKNEO, were
subsequently introduced by infection into these tat-
25 expressing cell lines as well as their parent cell line
lacking tat. Cell viability was then determined in
various concentrations of the HSVTK-specific cytotoxic
drug, acyclovir (ACV). The data are reported here as LD50
(the drug concentration at which 50% toxicity is
30 observed). The parental cell line containing the vector
but lacking tat (non-HIV-infected model) showed no
detectable toxicity by ACV at the concentrations tested
(see Figure 12). These cells thus require 100 uM ACV or
greater for cytotoxicity. This is true also for these
35 cells lacking the vectors. Thus the vectors alone, ACV
alone, or even the vector +ACV (solid boxes) is not
cytotoxic. However, cell lines which express HIV tat (the

experimental representation of an HIV infection) are
effectively killed by ACV. This is true to varying
degrees for all three vectors tested. These data indicate
that HIV-infected cells will be killed in the presence of
5 ACV and "potentiator" vectors.

In an analogous experiment, vectors KTVIHAX and
KTVIH5 Neo were introduced by infection into human T-cell
and monocyte cell lines Sup T1, HL60 and U937 cells.
Subsequently, these cells were infected with tat his or
10 αtat vectors, selected in histidinol, and cell viability
determined at various concentrations of the ACV analog,
FIAU. The LD_{50} reported in Table 1 (below) indicate that
a vector dependent increase in FIAU toxicity occurs in the
absence of HIV tat but is increased an additional ten- to
15 twentyfold when tat is present. This indicates that
although there is a baseline HSVTK expression in all but
HL60 cells, expression is even greater in the presence of
HIV tat.

TABLE 1

HIV tat inducibility of FIAU cytotoxicity in human monocyte and T-cell lines infected with conditionally lethal recombinant retroviral vectors

Cell Type	Vectors	tat	LD50FIAU(μM)
HL60 ("monocyte")	--	-	50
	--	+	50
	KTVIHAX	-	50
	KTVIHAX	+	<0.2
	KTVIH5Neo	-	50
	KTVIH5Neo	+	<0.2
U937 ("monocyte")	--	-	10
	KTVIHAX	-	0.5
	KTVIHAX	+	0.05
	KTVIH5Neo	-	0.5
	KTVIH5Neo	+	0.05
Sup T1 ("T-cell")	--	-	10
	--	+	5
	KTVIHAX	-	0.5
	KTVIHAX	+	0.05
	KTVIH5Neo	-	0.5
	KTVIH5Neo	+	0.05
H9 ("T-cell")	--	-	10
	KTVIHAX	-	2
	KTVIHAX	.+	0.2
	KTVIH5Neo	-	1
	KTVIH5Neo	+	0.05

Similarly, HIV infection of human T-cell line H9 +/- FIAU show a fivefold preferential inhibition (through cell killing) of HIV infection. Cultures were first treated with vector, then challenged with HIV for 4 days. Viral supernatants were then titred using the HIV assay, as described in Section IV.

In the case of HIV-infected cells, expression of the conditionally lethal HSVTK gene may be made even more HIV-specific by including cis-acting elements in the transcript ("CRS/CAR"), which require an additional HIV gene product, rev, for optimal activity (Rosen et al., Proc. Natl. Acad. Sci. USA 35:2071, 1988). Such a tat- and rev-responsive vector (RRKTVIH) has been constructed (see Figure 10) and amphotrophic virus has been generated.

More generally, cis elements present in mRNAs have been
shown in some cases to regulate mRNA stability or
translatability.   Sequences of this type (i.e., post-
transcriptional regulation of gene expression) may be used

5   for event- or tissue-specific regulation of vector gene
expression.   In addition, multimerization of these
sequences (i.e., rev-responsive "CRS/CAR" or tat-respon-
sive "TAR" elements for HIV) could result in even greater
specificity. It should be noted that this kind of condi-

10 tional activation of an inactive precursor into an active
product in cells may also be achieved using other viral
vectors with a shorter term effect, e.g., adenovirus
vectors.   Such vectors are capable of efficiently entering
cells and expressing proteins encoded by the vector over a

15 period of time from a couple of days to a month or so.
This period of time should be sufficient to allow killing
of cells which are infected by both HIV and the
recombinant virus, leading to HIV dependent activation of
expression of a gene carried by the recombinant virus.

20 This gene expression would then allow conversion of an
inactive precursor into an active (e.g., lethal) product.

Production, concentration and storage of vector
preparations is as previously described. Administration
is by direct in vivo administration as before or by ex

25 corpore treatment of PBL and/or bone marrow. Doses will
be at approximately the same levels as for Example 4.
Targeting of viral vector infection will not be through
the CD4 receptor, but may be accomplished through produc-
ing vector particles which will infect cells using the HIV

30 env protein (gp120) as a receptor.   Such HIV-tropic
viruses may be produced from an MLV-based packaging cell
line constructed from cells which have naturally high
levels of CD4 protein in their cell membrane (for example,
Sup T1 cells) or from any cell type "engineered" to

35 express the protein. The resultant virions, which form by
budding from the cell membrane itself, contain the CD4
protein in their membrane. Since membranes containing CD4

are known to fuse with membranes carrying HIV env, these virions should fuse with cells containing HIV env and result in the specific infection of HIV-infected cells which have gp120 on their surface. Such a packaging cell

5 line may require the presence of an MLV env protein to allow proper virion assembly and budding to result in infectious virions. If so, an MLV env which does not infect human cells (such as ecotropic env) would be used such that viral entry will occur only through the CD4/HIV

10 env interaction and not through the MLV env cell receptor, which would presumably not depend upon the presence of HIV-env for infection. Alternatively, the requirement for MLV env may be satisfied by a hybrid envelope where the amino-terminal binding domain has been replaced by the

15 amino-terminal HIV-env binding domain of CD4. This inversion of the normal virus-receptor interaction can be used for all types of viruses whose corresponding cellular receptor has been identified.

     In a similar manner to the preceding embodiment,
20 the retroviral vector construct can carry a gene for phosphorylation, phosphoribosylation, ribosylation, or other metabolism of a purine- or pyrimidine-based drug. This gene may have no equivalent in mammalian cells and might come from organisms such as a virus, bacterium,
25 fungus, or protozoan. An example of this would be the E. coli guanine phosphoribosyl transferase gene product, which is lethal in the presence of thioxanthine (see Besnard et al., Mol. Cell. Biol. 7:4139-4141, 1987). Conditionally lethal gene products of this type have
30 potential application to many presently known purine- or pyrimidine-based anticancer drugs, which often require intracellular ribosylation or phosphorylation in order to become effective cytotoxic agents. The conditionally lethal gene product could also metabolize a nontoxic drug,
35 which is not a purine or pyrimidine analogue, to a cytotoxic form (see Searle et al., Brit. J. Cancer 53:377-384, 1986).

Mammalian viruses in general tend to have "immediate early" genes which are necessary for subsequent transcriptional activation from other viral promoter elements. Gene products of this nature are excellent candidates for intracellular signals (or "identifying agents") of viral infection. Thus, conditionally lethal genes from transcriptional promoter elements responsive to these viral "immediate early" gene products could specifically kill cells infected with any particular virus. Additionally, since the human α and β interferon promoter elements are transcriptionally activated in response to infection by a wide variety of nonrelated viruses, the introduction of vectors expressing a conditionally lethal gene product like HSVTK, for example, from these viral-responsive elements (VRE) could result in the destruction of cells infected with a variety of different viruses.

In a fourth embodiment, the recombinant retrovirus carries a gene specifying a product which is not in itself toxic, but when processed or modified by a protein, such as a protease specific to a viral or other pathogen, is converted into a toxic form. For example, the recombinant retrovirus could carry a gene encoding a proprotein for ricin A chain, which becomes toxic upon processing by the HIV protease. More specifically, a synthetic inactive proprotein form of the toxic ricin or diphtheria A chains could be cleaved to the active form by arranging for the HIV virally encoded protease to recognize and cleave off an appropriate "pro" element.

In a fifth embodiment, the retroviral construct may express a "reporting product" on the surface of the target cells in response to the presence of an identifying agent in the cells (such as HIV tat protein). This surface protein can be recognized by a cytotoxic agent, such as antibodies for the reporting protein or by cytotoxic T-cells. In a similar manner, such a system can be used as a detection system (see below) to simply

identify those cells having a particular gene which expresses an identifying protein, such as the HIV tat gene.

Similarly, in a sixth embodiment, a surface
5 protein could be expressed which would itself be therapeutically beneficial. In the particular case of HIV, expression of the human CD4 protein specifically in HIV-infected cells may be beneficial in two ways:

1. Binding of CD4 to HIV env intracellularly
10 could inhibit the formation of viable viral particles much as soluble CD4 has been shown to do for free virus, but without the problem of systematic clearance and possible immunogenicity, since the protein will remain membrane bound and is structurally identical to endogenous CD4 (to
15 which the patient should be immunologically tolerant).

2. Since the CD4/HIV env complex has been implicated as a cause of cell death, additional expression of CD4 (in the presence of excess HIV-env present in HIV-infected cells) leads to more rapid cell death and thus
20 inhibits viral dissemination. This may be particularly applicable to monocytes and macrophages, which act as a reservoir for virus production as a result of their relative refractility to HIV-induced cytotoxicity (which, in turn, is apparently due to the relative lack of CD4 on
25 their cell surfaces).

EXAMPLE 6
Construction of p4TVIHAX retroviral vector
(see Figure 13)

30   1. The 9.4 kb Asu II/Xho I fragment was isolated from pN2.

2. The 0.6 kb Xho I/Eco R1 HIV promoter fragment was isolated from pSKHL.

3. The 1.4 kb coding region for human CD4 was
35 isolated as an Eco R1/Bst Y1 (Xho II) fragment from the expression vector, pMV7T4.

4.   The (A)n signal of HSVTK was isolated as a 0.3 kb Apa I/Sma I fragment, 3' repaired with T4 polymerase and dNTP's and cloned into the Sma I site of pUC31. After transforming bacteria, clones were screened

5   for orientation by restriction enzyme analysis (p31[A]n[+/-]). The 0.3 kb (A)n signal was then isolated as a 0.3 kb Bgl II/Acc I fragment.

5.   1-4 clones were mixed, ligated, used to transform bacteria and clones were identified by

10 restriction enzyme analysis.

Recombinant amphotrophic retroviruses have been produced and introduced into human monocyte and T-cell lines lacking or containing the HIV tat expression vector, tathis. Syncytia assays with HIV env-expressing mouse

15 fibroblasts show that monocyte cell lines HL60 and U937 themselves lack sufficient CD4 to fuse with these cells. However, HL60 and U937 cells containing vector 4TVIHAX can fuse with the reporter cells (HIV-env expressing cells) when HIV tat is present, but not in its absence. These

20 data indicate that CD4 expression is inducible and biologically active (as judged by syncytia formation). Experiments with the vector in human T-cell line, H9, indicated exceptionally high toxicity due to HIV infection and a correspondingly low HIV titre (more than 200-fold

25 lower than the HIV titre produced in H9 cells lacking the vector).

In a seventh embodiment, the retroviral vector codes for a ribozyme which will cleave and inactivate RNA molecules essential for viability of the vector infected

30 cell. By making ribozyme production dependent on an intracellular signal corresponding to the pathogenic state, such as HIV tat, toxicity is specific to the pathogenic state.

35 IV.   Immune Down-Regulation

As briefly described above, the present invention provides recombinant retroviruses which carry a

vector construct capable of suppressing one or more
elements of the immune system in target cells infected
with the retrovirus.

Specific down-regulation of inappropriate or
5   unwanted immune responses, such as in chronic hepatitis or
in transplants of heterologous tissue such as bone marrow,
can be engineered using immune-suppressive viral gene
products which suppress surface expression of trans-
plantation (MHC) antigen. Group C adenoviruses Ad2 and
10 Ad5 possess a 19 kd glycoprotein (gp 19) encoded in the E3
region of the virus. This gp 19 molecule binds to class I
MHC molecules in the endoplasmic reticulum of cells and
prevents terminal glycosylation and translocation of class
I MHC to the cell surface. For example, prior to bone
15 marrow transplantation, donor bone marrow cells may be
infected with gp 19-encoding vector constructs which upon
expression of the gp 19 inhibit the surface expression of
MHC class I transplantation antigens. These donor cells
may be transplanted with low risk of graft rejection and
20 may require a minimal immunosuppressive regimen for the
transplant patient. This may allow an acceptable donor-
recipient chimeric state to exist with fewer
complications. Similar treatments may be used to treat
the range of so-called autoimmune diseases, including
25 lupus erythromiatis, multiple sclerosis, rheumatoid
arthritis or chronic hepatitis B infection.

An alternative method involves the use of anti-
sense message, ribozyme, or other specific gene expression
inhibitor specific for T-cell clones which are
30 autoreactive in nature. These block the expression of the
T-cell receptor of particular unwanted clones responsible
for an autoimmune response. The anti-sense, ribozyme, or
other gene may be introduced using the viral vector
delivery system.

35

V.   Expression of Markers

        The above-described technique of expressing a palliative in a cell, in response to some identifying agent, can also be modified to enable detection of a
5 particular gene in a cell which expresses an identifying protein (for example, a gene carried by a particular virus), and hence enable detection of cells carrying that virus. In addition, this technique enables the detection of viruses (such as HIV) in a clinical sample of cells
10 carrying an identifying protein associated with the virus.

        This modification can be accomplished by providing a genome coding for a product, the presence of which can be readily identified (the "marker product"), and carrying a promoter, which responds to the presence of
15 the identifying protein in indicator cells, by switching expression of the reporting product between expressing and nonexpressing states. For example, HIV, when it infects suitable indicator cells, makes tat and rev. The indicator cells can thus be provided with a genome (such
20 as by infection with an appropriate recombinant retro-virus) which codes for a marker gene, such as the alkaline phosphatase gene, β-galactosidase gene or the luciferase gene, and a promoter, such as the HIV promoter, which controls expression of the marker gene. When the
25 indicator cells are exposed to a clinical sample to be tested, and the sample contains HIV, the indicator cells become infected with HIV, resulting in tat and/or rev expression (an identifying protein) therein. The HIV expression controls in the indicator cells would then
30 respond to tat and/or rev proteins by switching expression of genes encoding β-galactosidase, luciferase, or alkaline phosphatase (marker products) from normally "off" to "on." In the case of β-galactosidase or alkaline phosphatase, exposing the cells to substrate analogues results in a
35 color or fluorescence change if the sample is positive for HIV. In the case of luciferase, exposing the sample to luciferin will result in luminescence if the sample is

positive for HIV.   For intracellular enzymes such as
β-galactosidase, the viral titre can be measured directly
by counting colored or fluorescent cells, or by making
cell extracts and performing a suitable assay.   For the
5 membrane bond form of alkaline phosphatase, virus titre
can also be measured by performing enzyme assays on the
cell surface using a fluorescent substrate.   For secreted
enzymes, such as an engineered form of alkaline
phosphatase, small samples of culture supernatant are
10 assayed for activity, allowing continuous monitoring of a
single culture over time.   Thus, different forms of this
marker system can be used for different purposes.   These
include counting active virus or sensitively and simply
measuring viral spread in a culture and the inhibition of
15 this spread by various drugs.

     Further specificity can be incorporated into the
preceding system by testing for the presence of the virus
either with or without neutralizing antibodies to that
virus.   For example, in one portion of the clinical sample
20 being tested, neutralizing antibodies to HIV may be
present; whereas in another portion there would be no
neutralizing antibodies.   If the tests were negative in
the system where there were antibodies and positive where
there were no antibodies, this would assist in confirming
25 the presence of HIV.

     Within an analogous system for an _in vitro_
assay, the presence of a particular gene, such as a viral
gene, may be determined in a cell sample.   In this case,
the cells of the sample are infected with a suitable
30 retroviral vector which carries the reporter gene linked
to the expression controls of the virus of interest.   The
reporter gene, after entering the sample cells, will
express its reporting product (such as β-galactosidase or
luciferase) only if the host cell expresses the
35 appropriate viral proteins.

     These assays are more rapid and sensitive, since
the reporter gene can express a greater amount of

reporting product than identifying agent present, which
results in an amplification effect. Example 7 describes a
representative technique for detecting a gene which
expresses an identifying protein.

5

EXAMPLE 7
HIV-Specific Marker System or Assay

A.   Constructs

10   Reporter constructs under the control of the HIV
expression system are shown in Figure 14 (a recombinant
retroviral vector) and in Figure 15 (a simple plasmid used
by transfection). The pieces of these preferred vector
and plasmid reporters were derived as follows:

15   The retroviral backbone was derived from the
construct pAFVXM (Krieger et al., Cell 38:384, 1984),
which had been linearized using Xho I and Cla I. SV2neo
was obtained from the plasmid pKoneo (Hanahan, unpubl.) by
isolation of the 1.8 kb Cla I fragment.

20   The HIV LTR was isolated as a 0.7 kb Hind III
fragment from the plasmid pC15CAT (Arya et al., Science
229:69, 1985). Beta-gal was obtained from the plasmid
pSP65 β-gal (Cepko, pers. comm.) as a Hind III-Sma I
fragment. A secreted form of human placental alkaline

25 phosphatase was produced by introduction of a universal
terminator sequence after amino-acid 489 of the cell
surface form of alkaline phosphatase (as described by
Berger et al., Gene 66:1, 1988). The secreted alkaline
phosphatase gene was isolated as a 1.3 kb Hind III to

30 Kpn I fragment. The CRS-CAR sequences from HIV env were
obtained by isolating the 2.1 kb Kpn I to Bam HI fragment
from HTLVIIIB/BH10R3 (Fisher et al., Science 233:655,
1986). This fragment was inserted into pUC31 linearized
by Bam HI, and Kpn I pUC31 is pUC19 (Yanisch-Perron

35 et al., Gene 33:103, 1985) with extra Xho I, Bgl II, Bssh
II and Nco I sites between the Eco R1 and Kpn I sites of
pUC19. The Bam HI site of the resulting construct was

converted to a Nco I site to allow resection of the CRS-CAR sequences by Nco I digestion. The SV40 t intron was obtained from pSVOL (de Wet et al., _Mol. Cell. Biol._ _7_:725, 1987) as a 0.8 kb Nco I to Bam HI fragment.

5

B.   _Indicator Cells and Retroviral Vectors_

Human T-cell (H-9, CEM and Sup T1) and monocyte (U-937) cell lines were obtained from ATCC, and maintained in RPM1 1640 medium supplemented with 10% fetal bovine
10 serum and 1% penicillin/streptomycin.

The nonretroviral vectors were introduced into cell lines by electroporation using a Bio-Rad Gene Pulser. The cell lines were selected in G-418 (1 mg/ml) for 2-3 weeks to obtain stable G-418$_R$ cell lines, and then
15 dilution cloned to obtain clonal cell lines.

The pAF vectors were transfected into the PA317 packaging cell line as a calcium phosphate precipitate (Wigler et al., _Cell_ _16_:777, 1979). The virus-producing PA317 cells were co-cultivated with human monocyte cell
20 lines for 24 hours in the presence of polybrene, after which the suspension cells were removed and selected in G-418 and subcloned as above.

C.   _Assay_
25   Stable cell lines were infected with HIV (HTLV III$_B$) and the cells (β-gal) or media (alkaline phosphatase) assayed on a daily basis for 6 days post-infection.

β-Galactosidase Assay
30   Infected cells could be assayed by either: (i) _In situ_ histochemical staining as described by MacGregor et al. _Somatic Cell and Mol. Genetics_ _13_:253, 1987); or (ii) by using cell extracts in a solution enzymatic assay with ONPG as a substrate (Norton and
35 Coffin, _Mol. Cel. Biol._ _5_:281, 1985).

Soluble Alkaline Phosphatase Assay

Medium was removed from infected cells, microfuged for 10 seconds, and then heated to 68°C for 10 minutes to destroy endogenous phosphatases. The medium
5 was then microfuged for 2 minutes and an aliquot (10-50 µl) removed for assay. 100 µl of buffer (1 M diethanolamine, pH 9.8; 0.5 Mm $MgCl_2$; 10 mM L-homoarginine) was added and then 20 µl of 120 mM p-nitrophenylphosphate (in buffers) was added. The A_{405}
10 of the reaction mixture was monitored using an automatic plate reader.

Figures 16 and 17 depict typical results of a time course of infection of Sup T1 cells using the alkaline phosphatase assay in the presence of varying
15 concentrations of antiviral drugs. The "+" and "-" on day 6 indicate the presence or absence of syncytia.

The present invention provides a number of other techniques (described below) which can be used with the retroviral vector systems employed above, so as to enhance
20 their performance. Alternatively, these techniques may be used with other gene-delivery systems.

VI.   Packaging Cell Selection

This aspect of the present invention is based,
25 in part, upon the discovery of the major causes of low recombinant virus titres from packaging cells, and of techniques to correct those causes. Basically, at least five factors may be postulated as causes for low recombinant virus titres:
30   1.   the limited availability of viral packaging proteins;

          2.   the limited availability of retroviral vector RNA genomes;

          3.   the limited availability of cell membrane
35 for budding of the recombinant retroviruses;

          4.   the limited intrinsic packaging efficiency of the retroviral vector genome; and

5.   the density of the receptor specific for the envelope of a given retrovirus.

As noted above, the limited availability of viral packaging proteins is the initial limiting factor in
5   recombinant retrovirus production from packaging cells. When the level of packaging protein in the packaging cells is increased, titre increases to about 10^5 infectious units/milliliter, following which increasing packaging protein level has no further effect on titres.   However,
10 titres can be further augmented by also increasing the level of retroviral vector genome available for packaging. Thus, as described herein, it is advantageous to select producer cells that manufacture the maximum levels of packaging proteins and retroviral vector genomes.   It has
15 been discovered that the methods of identifying, and thus selecting, packaging cells and producer cells, described earlier under the section entitled "Background of the Invention," tend to lead to selection of many producer cells which produce low titres for the reasons described
20 below.

The present invention takes advantage of the previously disadvantageous fact that the protein expression level of a gene downstream from the 5' LTR or other promoter, and spaced therefrom by an intervening
25 gene, is substantially less than if the intervening gene were absent.   In the present invention, the selectable gene is placed downstream from a gene of the packaging genome or the gene of interest carried by the vector construct, but is still transcribed under the control of
30 the viral 5' LTR or other promoter without any splice donor or splice acceptor sites.   This accomplishes two things.   First, since the packaging genes or genes of interest are now upstream with no intervening gene between themselves and the promoter, their corresponding proteins
35 (packaging protein or protein of interest) will be expressed at a higher level (five- to twentyfold) than the selectable protein.   Second, the selectable protein will

be expressed on average at a lower level, with the
distribution of level of expression shifting toward lower
levels. In the case of the phleo^r protein, this shift in
distribution is illustrated by the broken curve indicated
5   in Figure 18. However, the selection level for resistance
to phleomycin remains the same, so that only the top-end
expressing cells survive. The levels of the packaging
protein or of the protein of interest will still be
proportional, only in this case, a higher level of
10 selectable protein corresponds to a much higher level of
packaging protein or protein of interest.

        Preferably, the foregoing procedure is performed
using a plasmid carrying one of the proviral gag/pol or
env packaging genes, along with a first selectable gene.
15 These cells are then screened for the cells producing the
highest levels of protein by reaction with an antibody
against env (or possibly gag/pol), a second fluorescent
antibody, and then sorted on a fluorescence-activated cell
sorter (FACS). Alternatively, other tests for protein
20 level may be used. Subsequently, the procedure and
screening are repeated using those selected cells, and the
other of the gag/pol or env packaging genes. In this
step, a second selectable gene (different from the first)
would be required downstream from the packaging gene and
25 the cells producing the largest amount of the second viral
protein selected. The procedure and screening are then
repeated using the surviving cells, with a plasmid
carrying the proviral vector construct bearing the gene of
interest and a third selectable gene, different from the
30 first or second selectable gene. As a result of this
procedure, cells producing high titres of the desired
recombinant retrovirus will be selected, and these can be
cultured as required to supply recombinant retrovirus. In
addition, gag and pol can be independently introduced and
35 selected.

        Example 8 describes the construction of gag/pol
and env plasmids designed to use these procedures.

EXAMPLE 3

Plasmids Designed to Make High Levels
of Packaging Proteins (Figure 19)

5   1.   The 2.7 kb Xba I fragment from pPAM (Miller
et al., Mol. Cell. Biol. 5:431, 1985), which contains the
amphotrophic env segment, was cloned in pUC18 at the Xba I
site, then removed with Hind III and Sma I. This fragment
was cloned into the vector pRSV neo (Gorman et al., Mol.
10   Cell. Biol. 2:1044, 1982; Southern et al., J. Mol. Appl.
Genet. 1:327, 1982) cut with Hind III and Pvu II, to give
pRSV env. A 0.7 kb Bam HI to BstE II fragment from the
plasmid pUT507 (Mulsant et al., Somat. Cell. Mol. Genet.
14:243, 1988) with the BstE II end filled in carries the
15   phleo resistance coding sequence. The 4.2 kb Bam HI to
Xho I fragment, the contiguous 1.6 kb Xho I to Xba I
(Xba I filled in) from RSVenv, and the phleo fragment were
ligated to give pRSVenv-phleo.

           2.   A   fragment   from   the   Pst I   site   at
20   nucleotide 563 of MLV (RNA Tumor Viruses, Vol. II, Cold
Spring Harbor, 1985) to the Sca I site at 5870 was derived
from pMLV-K (Miller et al., 1985, op. cit.) and cloned in
the Pst I to Bam HI (Bam HI filled-in) fragment from p4aA8
(Jolly et al., Proc. Natl. Acad. Sci. USA 80:477, 1983)
25   that   has   the   SV40   promoter,   the   pBR322   ampicillin
resistance and origin of replication and the SV40 poly A
site. This gives pSVgp. pSVgpDHFR was made using the
following fragments: the 3.6 kb Hind III to Sal I
fragment from pSVgp containing the SV40 promoter plus MLV
30   gag and some pol sequences; the 2.1 kb Sal I to Sca I
fragment from pMLV-K with the rest of the pol gene, the
3.2 kb Xba I (Xba I filled-in) to Pst I fragment from
pF400 with the DHFR gene plus poly A site, pBR322 origin
and half the ampicillin resistance gene; the 0.7 kb Pst I
35   to Hind III fragment from pBR322 with the other half of
the ampicillin resistance gene. This gives pSVgp-DHFR.
All these constructs are shown in Figure 19. These

plasmids can be transfected into 3T3 cells or other cells and high levels of gag, pol or env obtained.

       An additional method for accomplishing selection is to use a gene selection in one round and its antisense
5   in a subsequent round. For example, gag/pol may be introduced into an HPRT-deficient cell with the HPRT gene and selected for the presence of this gene using that media which requires HPRT for the salvage of purines. In the next round, the antisense to HPRT could be delivered
10 downstream to env and the cell selected in 6 thioguanine for the HPRT-deficient phenotype. Large amounts of antisense HPRT would be required in order to inactivate the HPRT gene transcripts, assuming no reversion occurred.

       In addition to the gag/pol expressing constructs
15 which begin at nucleotide 563 of MoMLV, several others can be constructed which contain upstream lead sequences. It has been observed by Prats et al. (RNA Tumor Viruses Meeting, Cold Spring Harbor, N.Y., 1988) that a glycosylated form of the gag protein initiates at
20 nucleotide 357 and a translation enhancer maps in the region between nucleotides 200-270. Therefore, gag/pol expressing constructs may be made beginning at the Bal I site (nucleotide 212) or Eag I site (nucleotide 346) to include these upstream elements and enhance vector
25 production.

       Envelope Substitutions

       The ability to express gag/pol and env function separately allows for manipulation of these functions
30 independently. A cell line that expresses ample amounts of gag/pol can be used, for example, to address questions of titre with regard to env. One factor resulting in low titres is the density of appropriate receptor molecules on the target cell or tissue. A second factor is the
35 affinity of the receptor for the viral envelope protein. Given that env expression is from a separate unit, a variety of envelope genes (requiring different receptor

proteins), such as xenotropic, polytropic, or amphotrophic
envs from a variety of sources, can be tested for highest
titres on a specific target tissue.   Furthermore,
envelopes from nonmurine retrovirus sources can be used

5 for pseudotyping a vector.   The exact rules for
pseudotyping (i.e., which envelope proteins will interact
with the nascent vector particle at the cytoplasmic side
of the cell membrane to give a viable viral particle
(Tato, Virology 88:71, 1978) and which will not (Vana,

10 Nature 336:36, 1988), are not well characterized.
However, since a piece of cell membrane buds off to form
the viral envelope, molecules normally in the membrane are
carried along on the viral envelope.   Thus, a number of
different potential ligands can be put on the surface of

15 viral vectors by manipulating the cell line making gag and
pol in which the vectors are produced or choosing various
types of cell lines with particular surface markers.   One
type of surface marker that can be expressed in helper
cells and that can give a useful vector-cell interaction

20 is the receptor for another potentially pathogenic virus.
The pathogenic virus displays on the infected cell surface
its virally specific protein (e.g., env) that normally
interacts with the cell surface marker or receptor to give
viral infection.   This reverses the specificity of the

25 infection of the vector with respect to the potentially
pathogenic virus by using the same viral protein-receptor
interaction, but with the receptors on the vector and the
viral protein on the cell.

It may be desirable to include a gene which

30 encodes for an irrelevant envelope protein which does not
lead to infection of target cells by the vector so
produced, but does facilitate the formation of infectious
viral particles.   For example, one could use human Sup T1
cells as a helper line.   This human T-cell line expresses

35 CD4 molecules at high levels on its surface.   Conversion
of this into a helper line can be achieved by expressing
gag/pol with appropriate expression vectors and also, if

necessary, the Moloney ecotropic env gene product as an
irrelevant (for human cells) envelope protein (the Moloney
ecotropic env only leads to infection of mouse cells).
Vectors produced from such a helper line would have CD4
5   molecules on their surfaces and are capable of infecting
only cells which express HIV env, such as HIV-infected
cells.

        In addition, hybrid envelopes (as described
below) can be used in this system as well, to tailor the
10   tropism (and effectively increase titres) of a retroviral
vector. A cell line that expresses ample amounts of a
given envelope gene can be employed to address questions
of titre with regard to gag and pol.

15   Cell Lines

        The most common packaging cell lines used for
MoMLV vector systems (psi2, PA12, PA317) are derived from
murine cell lines. There are several reasons why a murine
cell line is not the most suitable for production of human
20   therapeutic vectors:

        1.   They are known to contain endogenous
retroviruses.

        2.   They contain nonretroviral or defective
retroviral sequences that are known to package
25   efficiently.

        3.   There may be deleterious effects caused by
the presence of murine cell membrane components.

        Several non-murine cell lines are potential
packaging lines. These include Vero cells which are used
30   in Europe to prepare polio vaccine, WI38 which are used in
the U.S. in vaccine production, CHO cells which are used
in the U.S. for TPA preparation and D17 or other dog cells
that may have no endogenous viruses.

        Although the factors that lead to efficient
35   infection of specific cell types by retroviral vectors are
not completely understood, it is clear that because of
their relatively high mutation rate, retroviruses may be

adapted for markedly improved growth in cell types in
which initial growth is poor, simply by continual
reinfection and growth of the virus in that cell type (the
adapter cell). This can also be achieved using viral
5   vectors that encode some viral functions (e.g., env), and
which are passed continuously in cells of a particular
type which have been engineered to have the functions
necessary to complement those of the vector to give out
infectious vector particles (e.g., gag/pol). For example,
10 one can adapt the murine amphotropic virus 4070A to human
T-cells or monocytes by continuous growth and reinfection
of either primary cell cultures or permanent cell lines
such as Sup T1 (T-cells) or U937 (monocytes). Once
maximal growth has been achieved, as measured by reverse
15 transcriptase levels or other assays of virus production,
the virus is cloned out by any of a number of standard
methods, the clone is checked for activity (i.e., the
ability to give the same maximal growth characteristic on
transfection into the adapter cell type) and this genome
20 used to make defective helper genomes and/or vectors which
in turn, in an appropriately manufactured helper or
producer line, will lead to production of viral vector
particles which infect and express in the adapter cell
type with high efficiency (10^8 - 10^9 infectious units/ml).
25

VII. <u>Alternative Viral Vector Packaging Techniques</u>
        Two additional alternative systems can be used
to produce recombinant retroviruses carrying the vector
construct. Each of these systems takes advantage of the
30 fact that the insect virus, baculovirus, and the mammalian
viruses, vaccinia and adenovirus, have been adapted
recently to make large amounts of any given protein for
which the gene has been cloned. For example, see Smith
et al. (<u>Mol. Cell. Biol.</u> <u>3</u>:12, 1983); Piccini et al.
35 (<u>Meth. Enzymology</u>, <u>153</u>:545, 1987); and Mansour et al.
(<u>Proc. Natl. Acad. Sci. USA</u> <u>32</u>:1359, 1985).

These viral vectors can be used to produce
proteins in tissue culture cells by insertion of appro-
priate genes into the viral vector and, hence, could be
adapted to make retroviral vector particles.

5   Adenovirus vectors are derived from nuclear
replicating viruses and can be defective. Genes can be
inserted into vectors and used to express proteins in
mammalian cells either by _in vitro_ construction (Ballay et
al., _EMBO J._ 4:3861, 1985) or by recombination in cells
10 (Thummel et al., _J. Mol. Appl. Genetics_ 1:435, 1982).

One preferred method is to construct plasmids
using the adenovirus Major Late Promoter (MLP) driving:
(1) gag/pol, (2) env, (3) a modified viral vector
construct. A modified viral vector construct is possible
15 because the U3 region of the 5' LTR, which contains the
viral vector promoter, can be replaced by other promoter
sequences (see, for example, Hartman, _Nucl. Acids Res._
16:9345, 1988). This portion will be replaced after one
round of reverse transcriptase by the U3 from the 3' LTR.

20   These plasmids can then be used to make
adenovirus genomes _in vitro_ (Ballay et al., op. cit.), and
these transfected in 293 cells (a human cell line making
adenovirus E1A protein), for which the adenoviral vectors
are defective, to yield pure stocks of gag/pol, env and
25 retroviral vector carried separately in defective
adenovirus vectors. Since the titres of such vectors are
typically 10^7-10^{11}/ml, these stocks can be used to infect
tissue culture cells simultaneously at high multiplicity.
The cells will then be programmed to produce retroviral
30 proteins and retroviral vector genomes at high levels.
Since the adenovirus vectors are defective, no large
amounts of direct cell lysis will occur and retroviral
vectors can be harvested from the cell supernatants.

Other viral vectors such as those derived from
35 unrelated retroviral vectors (e.g., RSV, MMTV or HIV) can
be used in the same manner to generate vectors from
primary cells. In one embodiment, these adenoviral

vectors are used in conjunction with primary cells, giving rise to retroviral vector preparations from primary cells.

In an alternative system (which is more truly extracellular), the following components are used:

5   1. gag/pol and env proteins made in the baculovirus system in a similar manner as described in Smith et al. (supra) (or in other protein production systems, such as yeast or E. coli);

           2. viral vector RNA made in the known T7 or
10 SP6 or other in vitro RNA-generating system (see, for example, Flamant and Sorge, J. Virol. 62:1827, 1988);

           3. tRNA made as in (2) or purified from yeast or mammalian tissue culture cells;

           4. liposomes (with embedded env protein); and
15   5. cell extract or purified necessary components (when identified) (typically from mouse cells) to provide env processing, and any or other necessary cell-derived functions.

           Within this procedure (1), (2) and (3) are
20 mixed, and then env protein, cell extract and pre-liposome mix (lipid in a suitable solvent) added. It may, however, be necessary to earlier embed the env protein in the liposomes prior to adding the resulting liposome-embedded env to the mixture of (1), (2), and (3). The mix is
25 treated (e.g., by sonication, temperature manipulation, or rotary dialysis) to allow encapsidation of the nascent viral particles with lipid plus embedded env protein in a manner similar to that for liposome encapsidation of pharmaceuticals, as described in Gould-Fogerite et al.,
30 Anal. Biochem. 148:15, 1985). This procedure allows the production of high titres of replication incompetent recombinant retroviruses without contamination with pathogenic retroviruses or replication-competent retroviruses.

35

VIII. Cell Line-Specific Retroviruses - "Hybrid Envelope"

The host cell range specificity of a retrovirus
is determined in part by the env gene products. For
example, Coffin, J. (RNA Tumor Viruses 2:25-27, Cold
5   Spring Harbor, 1985) notes that the extracellular
component of the proteins from murine leukemia virus (MLV)
and Rous Sarcoma virus (RSV) are responsible for specific
receptor binding. The cytoplasmic domain of envelope
proteins, on the other hand, are understood to play a role
10   in virion formation. While pseudotyping (i.e., the
encapsidation of viral RNA from one species by viral
proteins of another species) does occur at a low
frequency, the envelope protein has some specificity for
virion formation of a given retrovirus. The present
15   invention recognizes that by creating a hybrid env gene
product (i.e., specifically, an env protein having
cytoplasmic regions and exogenous binding regions which
are not in the same protein molecule in nature) the host
range specificity may be changed independently from the
20   cytoplasmic function. Thus, recombinant retroviruses can
be produced which will specifically bind to preselected
target cells.

In order to make a hybrid protein in which the
receptor binding component and the cytoplasmic component
25   are from different retroviruses, a preferred location for
recombination is within the membrane-spanning region of
the cytoplasmic component. Example 9 describes the
construction of a hybrid env gene which expresses a
protein with the CD4 binding portion of the HIV envelope
30   protein coupled to the cytoplasmic domain of the MLV
envelope protein.

EXAMPLE 9

Hybrid HIV-MLV Envelopes

35   A hybrid envelope gene is prepared using
in vitro mutagenesis (Kunkel, Proc. Natl. Acad. Sci. USA
82:488-492, 1985) to introduce a new restriction site at

an appropriate point of junction. Alternatively, if the
two envelope sequences are on the same plasmid, they can
be joined directly at any desired point using _in vitro_
mutagenesis. The end result in either case is a hybrid
5   gene containing the 5' end of the HIV gp 160 and the 3'
end of MLV p15E. The hybrid protein expressed by the
resulting recombinant gene is illustrated in Figure 20 and
contains the HIV gp120 (CD4 receptor binding protein), the
extracellular portion of HIV gp 41 (the gp 120 binding and
10 fusigenic regions), and the cytoplasmic portion of MLV
p15E, with the joint occurring at any of several points
within the host membrane. A hybrid with a fusion joint at
the cytoplasmic surface (joint C in Figure 20) causes
syncytia when expressed in Sup T1 cells. The number of
15 apparent syncytia are approximately one-fifth that of the
nonhybrid HIV envelope gene in the same expression vector.
Syncytia with the hybrid occurs only when the rev protein
is co-expressed in trans. A hybrid with a fusion joint at
the extracellular surface (joint A in Figure 20) gives no
20 syncytia while hybrid B (in the middle of the
transmembrane regions) gives approximately five-fold less
syncytium on Sup T1 cells than hybrid C.

While Example 9 illustrates one hybrid protein
produced from two different retroviruses, the possibili-
25 ties are not limited to retroviruses or other viruses.
For example, the beta-receptor portion of human inter-
leukin-2 may be combined with the envelope protein of MLV.
In this case, a recombination would preferably be located
in the gp 70 portion of the MLV env gene, leaving an
30 intact p15E protein. Furthermore, the foregoing technique
may be used to create a recombinant retrovirus with an
envelope protein which recognizes antibody Fc segments.
Monoclonal antibodies which recognize only preselected
target cells only could then be bound to such a
35 recombinant retrovirus exhibiting such envelope proteins
so that the retrovirus would bind to and infect only those
preselected target cells.

The approach may also be used to achieve tumor-specific targeting and killing by taking advantage of three levels of retroviral vector specificity; namely, cell entry, gene expression, and choice of protein 5 expressed. Retroviral vectors enter cells and exert their effects at intracellular sites. In this respect their action is quite unique. Using this property, and the three levels of natural retroviral specificity (above), retroviral vectors may be engineered to target and kill 10 tumor cells.

The overall goal of targeting of retrovirus to tumor cells may be accomplished by two major experimental routes; namely, a) selection in tissue culture (or in animals) for retroviruses that grow preferentially in 15 tumor cells; or b) construction of retroviral vectors with tissue (tumor) -specific promoters with improvements being made by in vitro passage, and negative and positive drug-sensitivity selection.

At least four selective protocols may be 20 utilized to select for retrovirus which grow preferentially in tumor cells; namely, 1) "Env Selection by Passage In Vitro," wherein selection of retrovirus with improved replicative growth ability is accomplished by repeated passage in tumor cells; 2) "Selection with a Drug 25 Resistance Gene," wherein genetic selection for tumor "specific" retroviruses is based on viral constructs containing a linked drug resistance gene; 3) "Hybrid-Env," wherein selection (by protocol #1 or #2, above) of retrovirus with tumor-"specificity" is initiated from a 30 construct containing a hybrid envelope gene which is a fusion of a tumor receptor gene (i.e., an anti-tumor antibody H-chain V-region gene fused with env; or, a growth receptor fused with env); in this case selection begins at a favorable starting point, e.g., an env which 35 has some specificity for tumor cells; or 4) "Selection by Passage In Vitro and Counter Selection by Co-cultivation with Normal Cells," wherein growth in tumor cells is

selected-for by repeated passage in mixtures of drug-
resistant tumor cells and drug-sensitive normal cells.

     With respect to retroviral vector constructs
carrying tissue (tumor) -specific promoters, biochemical
5   markers with different levels of tissue-specificity are
well known, and genetic control through tissue-specific
promoters is understood in some systems. There are a
number of genes whose transcriptional promoter elements
are relatively active in rapidly growing cells (i.e.,
10 transferrin receptor, thymidine kinase, etc.) and others
whose promoter/enhancer elements are tissue specific
(i.e., HBV enhancer for liver, PSA promoter for prostate).
Retroviral vectors and tissue-specific promoters (present
either as an internal promoter or within the LTR) which
15 can drive the expression of selectable markers and cell
cycle genes (i.e., drug sensitivity, Eco gpt; or HSVTK in
TK-cells). Expression of these genes can be selected for
in media containing mycophenolic acid or HAT,
respectively. In this manner, tumor cells containing
20 integrated provirus which actively expresses the drug
resistance gene will survive. Selection in this system
may involve selection for both tissue-specific promoters
and viral LTRs. Alternatively, specific expression in
tumor cells, and not in normal cells, can be counter-
25 selected by periodically passaging virus onto normal
cells, and selecting against virus that express Eco gpt or
HSVtk (drug sensitivity) in those cells (by thioxanthine
or acyclovir). Infected cells containing integrated
provirus which express Eco gpt or tk phenotype will die
30 and thus virus in that cell type will be selected against.

IX. <u>Site-Specific Integration</u>

     Targeting a retroviral vector to a predetermined
locus on a chromosome increases the benefits of gene-
35 delivery systems. A measure of safety is gained by direct
integration to a "safe" spot on a chromosome, i.e., one
that is proven to have no deleterious effects from the

94

insertion of a vector. Another potential benefit is the ability to direct a gene to an "open" region of a chromosome, where its expression would be maximized. Two techniques for integrating retroviruses at specific sites 5 are described below.

(i)   Homologous Recombination

One technique for integrating an exogenous gene of a vector construct of a recombinant retrovirus into a 10 specific site in a target cell's DNA employs homologous recombination. Plasmids containing sequences of DNA of greater than about 300 bp that are homologous to genomic sequences have been shown to interact (either by replacement or insertion) with those genomic sequences at a rate 15 that is greater than 10^3-fold over a specific interaction in the absence of such homology (see Thomas and Capecchi, Cell 51:503-12, 1987; and Doetscheman et al., Nature 330:576-78, 1987). It has been shown that an insertion event, or alternatively, a replacement event, may be 20 driven by the specific design of the vector.

In order to employ homologous recombination in site-specific retroviral integration, a vector construct should be modified such that (a) homologous sequences (to the target cell's genome) are incorporated into the 25 construct at an appropriate location; and (b) the normal mechanism of integration does not interfere with the targeting occurring due to homologous sequences. A preferred approach in this regard is to add homologous sequences (greater than about 300 bp) in the 3' LTR, 30 downstream from the U3 inverted repeat. In this approach, the construct is initially made with a region of homology inserted in the 3' LTR at the Nhe 1 site in U3. Reverse transcription in the host cell will result in a duplication of the region of homology in the 5' LTR within 35 31 bp of the end of the inverted repeat (IR). Integration into the host genome will occur in the presence or absence of the normal integration mechanism. The gene in the

vector may be expressed, whether from the LTR or from an internal promoter. This approach has the effect of placing a region of homology near a potential free end of the double-stranded retrovirus vector genome. Free ends

5 are known to increase the frequency of homologous recombination by a factor of approximately ten. In this approach, it may be necessary to defeat the normal mechanism of integration, or to at least modify it to slow down the process, allowing time for homologous DNAs to

10 line up. Whether this latter modification is required in a particular case can be readily ascertained by one skilled in the art.

    (ii) Integrase Modification

15   Another technique for integrating a vector construct into specific, preselected sites of a target cell's genome involves integrase modification.

            The retrovirus pol gene product is generally processed into four parts: (i) a protease which processes

20 the viral gag and pol products; (ii) the reverse transcriptase; and (iii) RNase H, which degrades RNA of an RNA/DNA duplex; and (iv) the endonuclease or "integrase."

            The general integrase structure has been analyzed by Johnson et al. (Proc. Natl. Acad. Sci. USA

25 83:7648-7652, 1986). It has been proposed that this protein has a zinc binding finger with which it interacts with the host DNA before integrating the retroviral sequences.

            In other proteins, such "fingers" allow the

30 protein to bind to DNA at particular sequences. One illustrative example is the steroid receptors. In this case, one can make the estrogen receptor, responding to estrogens, have the effect of a glucocorticoid receptor, responding to glucocorticoids, simply by substituting the

35 glucocorticoid receptor "finger" (i.e., DNA binding segment) in place of the estrogen receptor finger segment in the estrogen receptor gene. In this example, the

position in the genome to which the proteins are targeted
has been changed. Such directing sequences can also be
substituted into the integrase gene in place of the
present zinc finger. For instance, the segment coding for
5   the DNA binding region of the human estrogen receptor gene
may be substituted in place of the DNA binding region of
the integrase in a packaging genome. Initially, specific
integration would be tested by means of an _in vitro_
integration system (Brown et al., _Cell_ 29:347-356, 1987).
10   To confirm that the specificity would be seen _in vivo_,
this packaging genome is used to make infectious vector
particles, and infection of and integration into estrogen-
sensitive and estrogen-nonsensitive cells compared in
culture.

15   Through use of this technique, incoming viral
vectors may be directed to integrate into preselected
sites on the target cell's genome, dictated by the genome-
binding properties of site-specific DNA-binding protein
segments spliced into the integrase genome. It will be
20   understood by those skilled in the art that the
integration site must, in fact, be receptive to the
fingers of the modified integrase. For example, most
cells are sensitive to glucocorticoids and hence their
chromatin has sites for glucocorticoid receptors. Thus,
25   for most cells, a modified integrase having a glucocorti-
coid receptor finger would be suitable to integrate the
proviral vector construct at those glucocorticoid
receptor-binding sites.

30   X.   Production of Recombinant Retroviral Vectors in
           Transgenic Animals
           Two problems previously described with helper
line generation of retroviral vectors are: (a) difficulty
in generating large quantities of vectors; and (b) the
35   current need to use permanent instead of primary cells to
make vectors. These problems can be overcome with
producer or packaging lines that are generated in

transgenic animals. These animals would carry the packaging genomes and retroviral vector genomes. Current technology does not allow the generation of packaging cell lines and desired vector-producing lines in primary cells

5 due to their limited life span. The current technology is such that extensive characterization is necessary, which eliminates the use of primary cells because of senescence. However, individual lines of transgenic animals can be generated by the methods provided herein which produce the

10 packaging functions, such as gag, pol or env. These lines of animals are then characterized for expression in either the whole animal or targeted tissue through the selective use of housekeeping or tissue-specific promoters to transcribe the packaging functions. The vector to be

15 delivered is also inserted into a line of transgenic animals with a tissue-specific or housekeeping promoter. As discussed above, the vector can be driven off such a promoter substituting for the U3 region of the 5' LTR (Figure 21). This transgene could be inducible or

20 ubiquitous in its expression. This vector, however, is not packaged. These lines of animals are then mated to the gag/pol/env animal and subsequent progeny produce packaged vector. The progeny, which are essentially identical, are characterized and offer an unlimited source

25 of primary producing cells. Alternatively, primary cells making gag/pol and env and derived from transgenic animals can be infected or transfected in bulk with retrovirus vectors to make a primary cell producer line. Many different transgenic animals or insects could produce

30 these vectors, such as mice, rats, chickens, swine, rabbits, cows, sheep, fish and flies. The vector and packaging genomes would be tailored for species infection specificity and tissue-specific expression through the use of tissue-specific promoters and different envelope

35 proteins. An example of such a procedure is illustrated in Figure 22.

Although the following examples of transgenic
production of primary packaging lines are described only
for mice, these procedures can be extended to other
species by those skilled in the art. These transgenic
5   animals may be produced by microinjection or gene transfer
techniques. Given the homology to MLV sequences in mice
genome, the final preferred animals would not be mice.

EXAMPLE 10
10   Production of Gag/Pol Proteins Using Housekeeping
Promoters for Ubiquitous Expression
in Transgenic Animals
An example of a well-characterized housekeeping
promoter is the HPRT promoter. HPRT is a purine salvage
15   enzyme which expresses in all tissues. (See Patel et al.,
Mol. Cell Biol. 6:393-403, 1986 and Melton et al., Proc.
Natl. Acad. Sci. 81:2147-2151, 1984). This promoter is
inserted in front of various gag/pol fragments (e.g.,
Bal I/Sca I; Aat II/Sca I; Pst I/Sca I of MoMLV; see RNA
20   Tumor Viruses 2, Cold Spring Harbor Laboratory, 1985) that
are cloned in Bluescript plasmids (Strategene, Inc.) using
recombinant DNA techniques (see Maniatis et al., Molecular
Cloning: A Laboratory Manual, Cold Spring Harbor, 1982).
The resulting plasmids are purified (Maniatis et al.,
25   op. cit.) and the relevant genetic information isolated
using Geneclean (Bio 101) or electroelution (see Hogan
et al. (eds.), Manipulating the Mouse Embryo: A
Laboratory Manual, Cold Spring Harbor, 1986).
These fully characterized DNAs are microinjected
30   in the pronucleus of fertilized mouse ova at a
concentration of 2 ug/ml. Live-born mice are screened by
tail blot analyses (see Hogan et al., op. cit.).
Transgenic-positive animals are characterized for
expression levels of gag-pol proteins by immunoprecipita-
35   tion of radiolabeled primary cells, such as fibroblast
(see Harlow et al. (eds.), Antibodies: A Laboratory
Manual, Cold Spring Harbor, 1988). Animals then bred to

homozygosity for establishment of animal lines that produce characterized levels of gag-pol.

EXAMPLE 11
Production of env Proteins/Hybrid Envelope Proteins
Using Housekeeping Promoters for Ubiquitous Expression
in Transgenic Animals

This example utilizes the HPRT promoter for expression of either envelope or hybrid envelope proteins. The envelope proteins can be from any retrovirus that is capable of complementing the relevant gag-pol, in this case that of MLV. Examples are ecotropic MLV, amphotrophic MLV, xenotropic MLV, polytropic MLV, or hybrid envelopes. As above, the envelope gene is cloned behind the HPRT promoter using recombinant DNA techniques (see Maniatis et al., op. cit.). The resulting "minigene" is isolated (see Hogan et al., op. cit.), and expression of envelope protein is determined (Harlow et al., op. cit.). The transgenic envelope animals are bred to homozygosity to establish a well-characterized envelope animal.

EXAMPLE 12
Production of gag-pol-env Animals Using Housekeeping
Promoters for Ubiquitous Expression
in Transgenic Animals

This uses the well-characterized gag-pol animals, as well as the animals for the establishment of a permanent gag-pol/envelope animal line. This involves breeding to homozygosity and the establishment of a well-characterized line. These lines are then used to establish primary mouse embryo lines that can be used for packaging vectors in tissue culture. Furthermore, animals containing the retroviral vector are bred into this line.

EXAMPLE 13

Production of Tissue-Specific Expression of gag-pol-env or Hybrid Envelope in Transgenic Animals

5   This example illustrates high level expression of the gag/pol, envelope, or hybrid envelope in specific tissues, such as T-cells. This involves the use of CD2 sequences (see Lang et al., EMBO J. 7:1675-1682, 1988) that give position and copy number independence. The
10 1.5 kb Bam HI/Hind III fragment from the CD2 gene is inserted in front of gag-pol, envelope, or hybrid envelope fragments using recombinant DNA techniques. These genes are inserted into fertilized mouse ova by microinjection. Transgenic animals are characterized as before.
15 Expression in T-cells is established, and animals are bred to homozygosity to establish well-characterized lines of transgenic animals. Gag-pol animals are mated to envelope animals to establish gag-pol-env animals expressing only in T-cells. The T-cells of these animals are then a
20 source for T-cells capable of packaging retroviral vectors. Again, vector animals can be bred into these gag-pol-env animals to establish T-cells expressing the vector.

          This technique allows the use of other tissue-
25 specific promoters, such as milk-specific (whey), pancre-atic (insulin or elastase), or neuronal (myelin basic protein) promoters. Through the use of promoters, such as milk-specific promoters, recombinant retroviruses may be isolated directly from the biological fluid of the
30 progeny.

EXAMPLE 14
Production of Either Housekeeping or Tissue-Specific Retroviral Vectors in Transgenic Animals

35   The insertion of retroviruses or retroviral vectors into the germ line of transgenic animals results in little or no expression. This effect, described by

Jaenisch (see Jahner et al., _Nature_ 298:623-628, 1982), is attributed to methylation of 5' retroviral LTR sequences. This technique would overcome the methylation effect by substituting either a housekeeping or tissue-specific

5   promoter to express the retroviral vector/retrovirus. The U3 region of the 5' LTR, which contains the enhancer elements, is replaced with regulatory sequences from housekeeping or tissue-specific promoters (see Figure 20). The 3' LTR is fully retained, as it contains sequences

10   necessary for polyadenylation of the viral RNA and integration. As the result of unique properties of retroviral replication, the U3 region of the 5' LTR of the integrated provirus is generated by the U3 region of the 3' LTR of the infecting virus. Hence, the 3' is

15   necessary, while the 5' U3 is dispensable. Substitution of the 5' LTR U3 sequences with promoters and insertion into the germ line of transgenic animals results in lines of animals capable of producing retroviral vector transcripts. These animals would then be mated to

20   gag-pol-env animals to generate retroviral-producing animals (see Figure 22).

From the foregoing it will be appreciated that, although specific embodiments of the invention have been

25   described herein for purposes of illustration, various modifications may be made without deviating from the spirit and scope of the invention. Accordingly, the invention is not limited except as by the appended claims.

30

Claims

1.   A recombinant retrovirus carrying a vector construct which directs the expression in target cells infected with the retrovirus of at least one protein or modified form thereof being capable of stimulating an immune response within an animal, the vector construct further comprising a segment encoding a viral capsid protein and a regulatory response element, and a segment encoding at least one regulatory element.

2.   The recombinant retrovirus of claim 1 wherein the viral capsid protein is the HIV gag protein, the regulatory element is the HIV rev protein, and the regulatory response element is the HIV rev responsive regulatory element.

3.   The recombinant retrovirus of claim 1 wherein the vector construct directs the expression of at least one HIV protein selected from the group consisting of HIV gag, HIV pol, HIV rev, HIV vpu, HIV vif, HIV and HIV prot.

4.   The recombinant retrovirus of claim 1 wherein the segment encoding the viral capsid protein is HIV gag, the segment being operably linked to a second gene, wherein the HIV gag and second gene are capable of directing the expression of the protein or modified form thereof.

5.   A recombinant retrovirus carrying a vector construct having a tissue tumor-specific transcriptional promoter/enhancer element, the construct directing the expression of a palliative in cells having a tissue type compatible with the promoter, said palliative being capable of inhibiting a function of a pathogenic agent necessary for pathogenicity.

6.   A recombinant retrovirus carrying a vector construct which directs the expression of a peptide having multiple epitopes, one or more of said epitopes being derived from different proteins.

7.   A recombinant retrovirus carrying a vector construct which directs the expression of peptidic antigenic fragment or modified form thereof in target cells infected with the retrovirus, said antigenic fragment or modified form thereof being capable of stimulating an immune response within an animal.

8. A recombinant retrovirus carrying a vector construct which directs the expression of at least one antigen or modified form thereof and an MHC protein or modified form thereof in target cells infected with the retrovirus, said antigen or modified form thereof and the MHC protein or modified form thereof being capable of stimulating an immune response within an animal.

9. A recombinant retrovirus carrying a vector construct which directs the expression of a first antigen or modified form thereof in target cells infected with the retrovirus, said first antigen being capable of stimulating an immune response within an animal against a second antigen or modified form thereof, wherein the first and the second antigens are related but not identical.

10. The recombinant retrovirus of claim 9 wherein the first and second antigens or modified forms thereof share at least 40% homology, but not more than 99% homology, in a sequence of 8 to 100 amino acids.

11. A recombinant retrovirus carrying a vector construct which directs the expression of at least one antigen or modified form thereof in target cells infected with the retrovirus, said antigen or modified form thereof being capable of stimulating an immune response *in vitro*.

12. A DNA vector construct which directs the expression of at least one antigen or modified form thereof in target cells transfected with the DNA, said antigen or modified form thereof being capable of stimulating an immune response *in vitro*.

13. The recombinant retrovirus of claims 11 or 12 wherein the *in vitro* immune response is a cell-mediated immune response.

14. The recombinant retrovirus of claims 11 or 12 wherein the *in vitro* immune response is a CTL response.

15. A recombinant retrovirus carrying a vector construct which directs the transient expression of at least one antigen or modified form thereof in target cells infected with the retrovirus, said antigen or modified form thereof being capable of stimulating an immune response within an animal.

16.   A DNA vector construct which directs the transient expression of at least one antigen or modified form thereof in target cells transfected with the DNA, said antigen or modified form thereof being capable of stimulating an immune response within an animal.

17.   A recombinant retrovirus carrying a vector construct which directs the expression of a palliative in cells infected with the retrovirus, the palliative being capable of inhibiting the activity of a protein produced by a pathogenic agent.

18.   A recombinant retrovirus carrying a vector construct which directs the expression of an RNA molecule which functions as a ribozyme specific for a pathogenic RNA molecule.

19.   The recombinant retrovirus of any of claims 1-18 wherein said retrovirus is replication defective.

20.   A method of producing a recombinant retrovirus, comprising:
packaging a vector construct in a capsid and envelope such that a replication defective recombinant retrovirus according to claim 19 is produced.

21.   *Ex vivo* cells infected with a recombinant retrovirus or transfected with a DNA vector construct according to any of claims 1-19.

22.   Eucaryotic cells infected with a recombinant retrovirus according to any of claims 1-11 and 13-18, said cells being capable of generating infectious particles containing any one of said vector constructs.

23.   A pharmaceutical composition comprising a retrovirus according to any one of claims 1-11 and 13-18, in combination with a physiologically acceptable carrier or diluent.

24.   The pharmaceutical composition of claim 21, for use as an active therapeutic substance.

25.   A method of identifying an antigen or antigenic epitopes in a protein, reactive with T lymphocytes, comprising:

(a)   preparing a multiplicity of different recombinant retroviruses each carrying a recombinant vector construct directing the expression of a different antigen, or peptidic fragment of the antigen;

(b)   infecting a multiplicity of target cells with the different recombinant retroviruses; and

(c)   testing for the ability of CTL or antibodies to kill target cells expressing the different antigens or peptidic fragments of the antigen, and therefrom determining which antigen or peptidic fragments of the antigen are reactive with the T lymphocytes.

-1/39-
RETROVIRAL CONSTRUCTS OF ENV^R

FIG.1
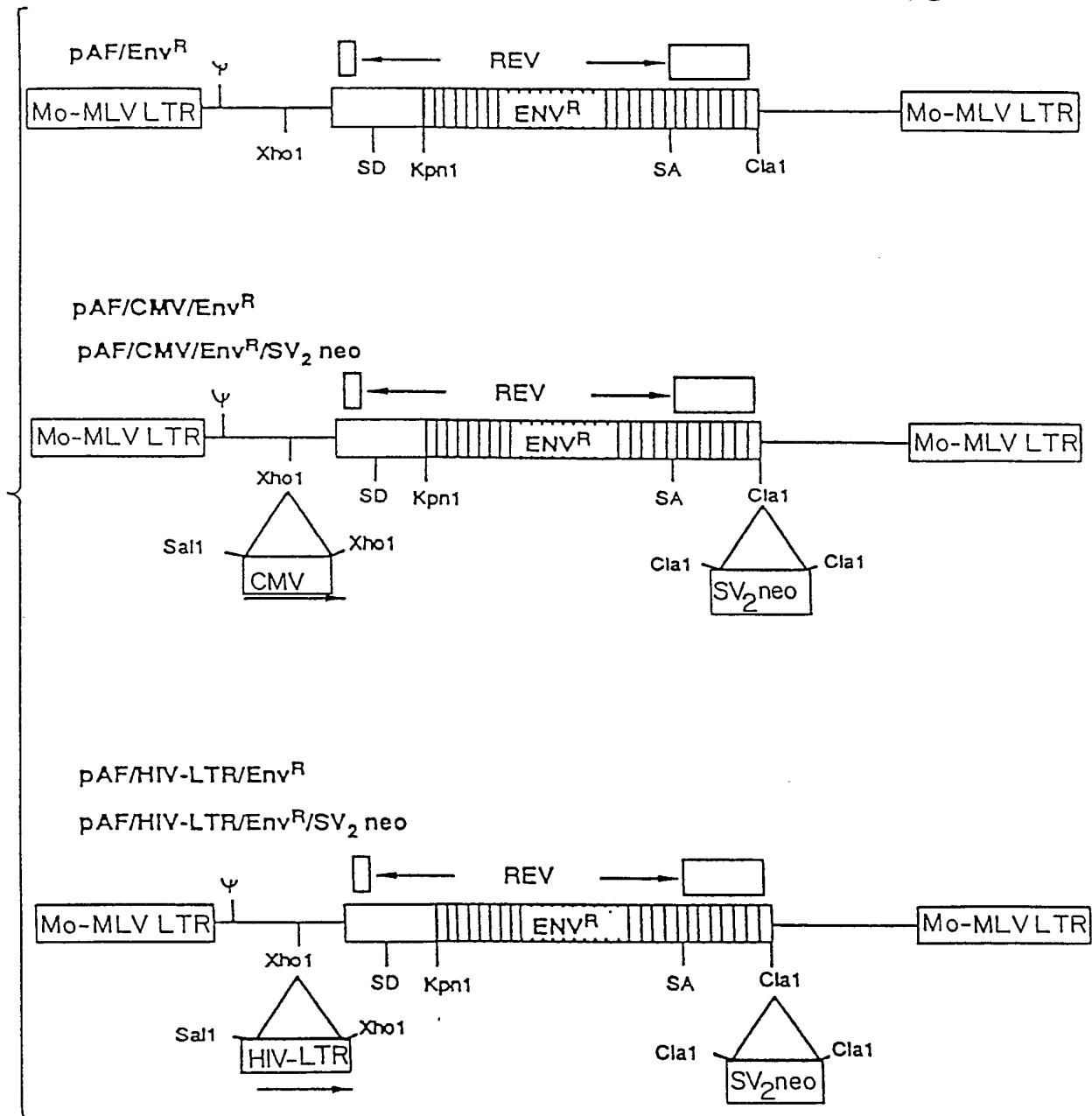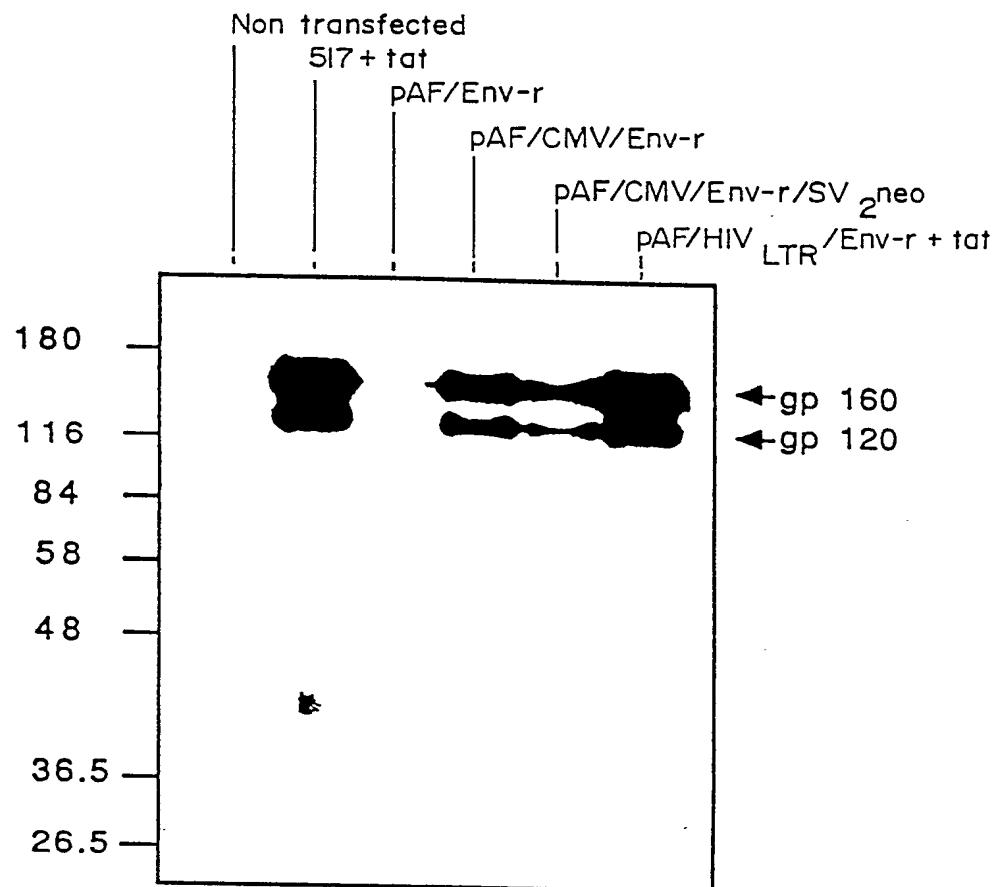
FIG.2

FIG. 3

Induction of Anti-HIV env CTL in Balb/c Mice

Using Retroviral-Infected Stimulator Cells

Balb/c (H-2^d) ---- Non-Primed Balb/c
Spleen cells
                    +B/C10MEenv-29 ---> ----> Spleen + B/C10MEenv-29   B/C10MEenv-29
                                              cells
                    +B/C10ME -------> ----> Spleen + B/C10ME   B/C10ME
4 x 10^7 cells, ip   cells
B/C10MEenv-29
(H-2^d)

PRIMING & BOOST   IN VITRO STIMULATION   ASSAY TARGET CELLS
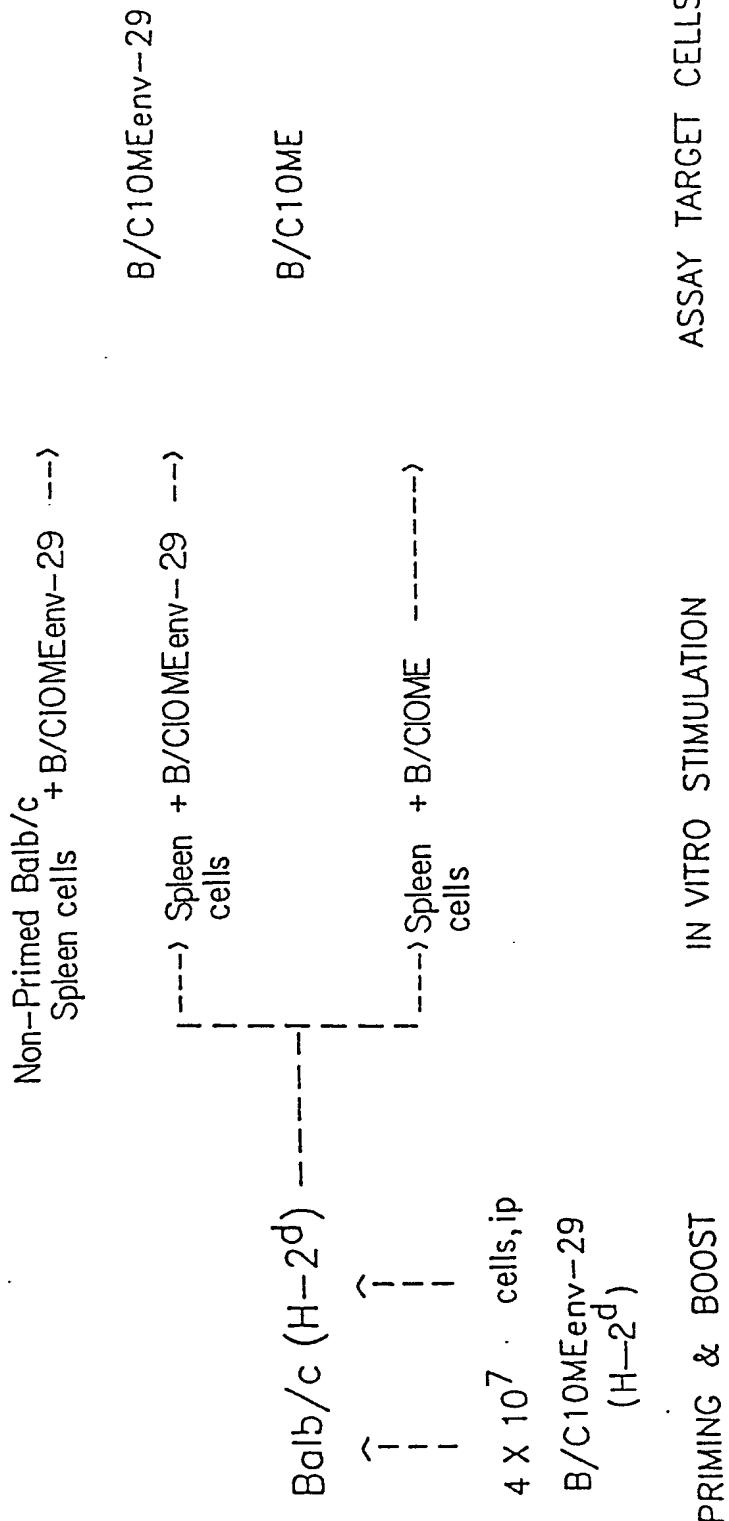
FIG. 4A

INDUCTION OF CYTOTOXIC EFFECTORS IN MICE IMMUNIZED WITH
BCenv STIMULATOR CELLS
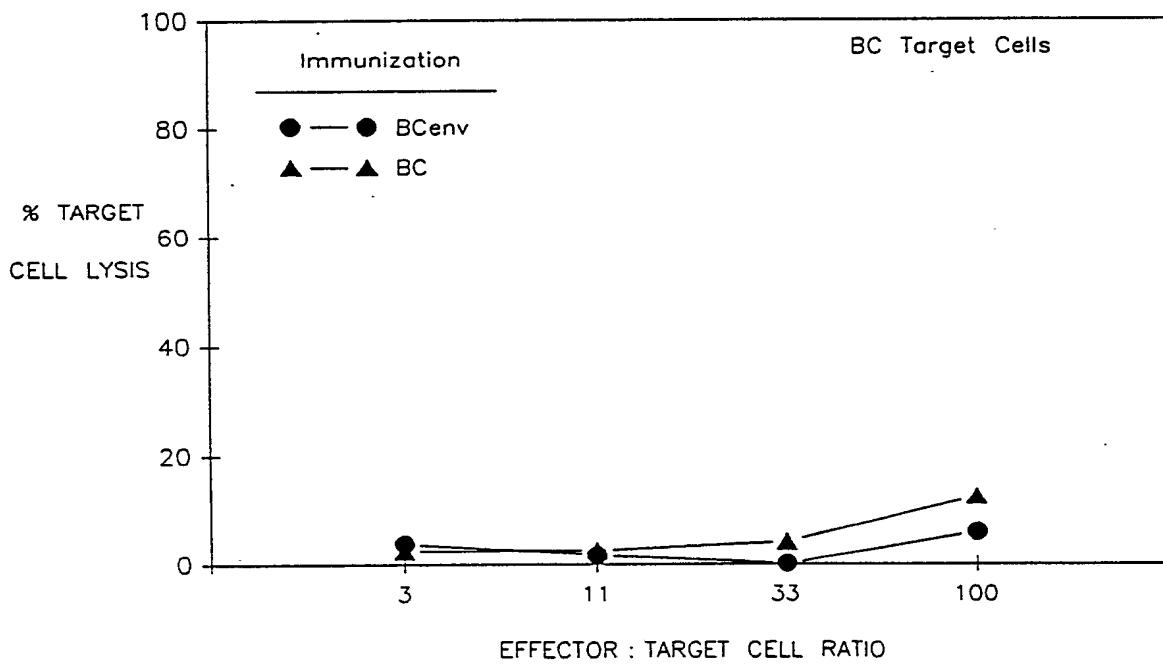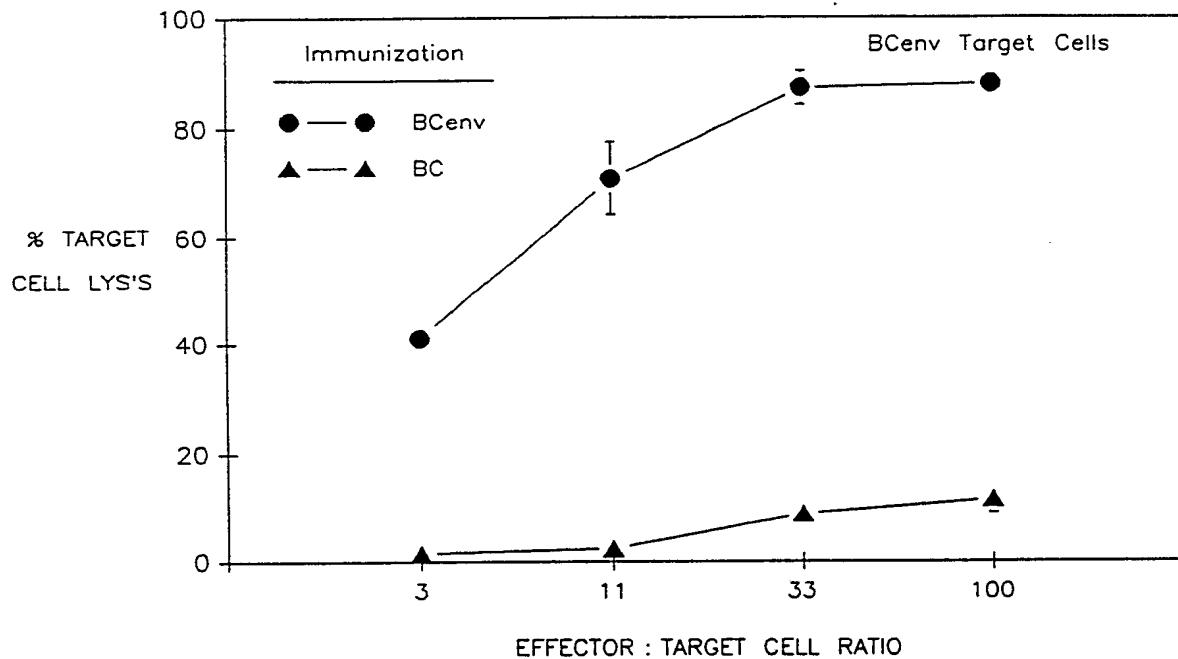
FIG. 4B

SPECIFICITY OF ANTI-BCenv CTL EFFECTORS
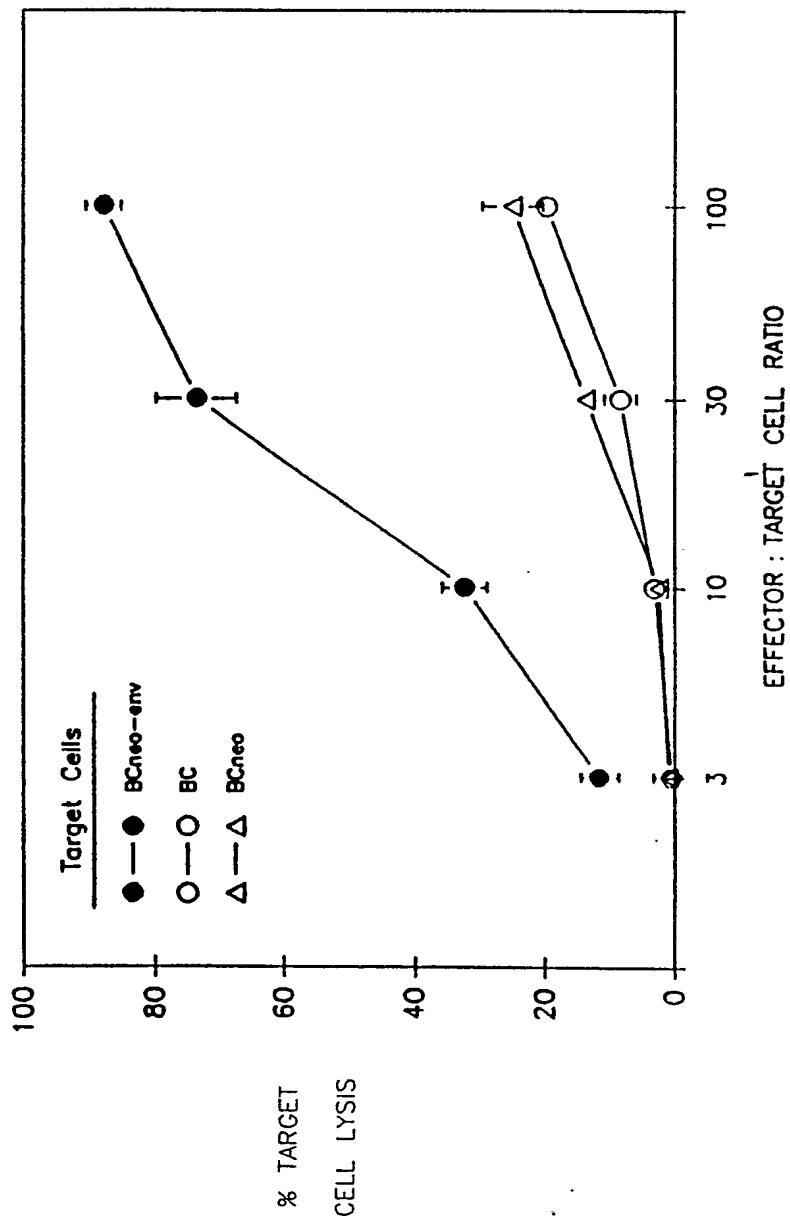
FIG. 4C

PHENOTYPE OF ANTI-BCenv EFFECTOR CELLS
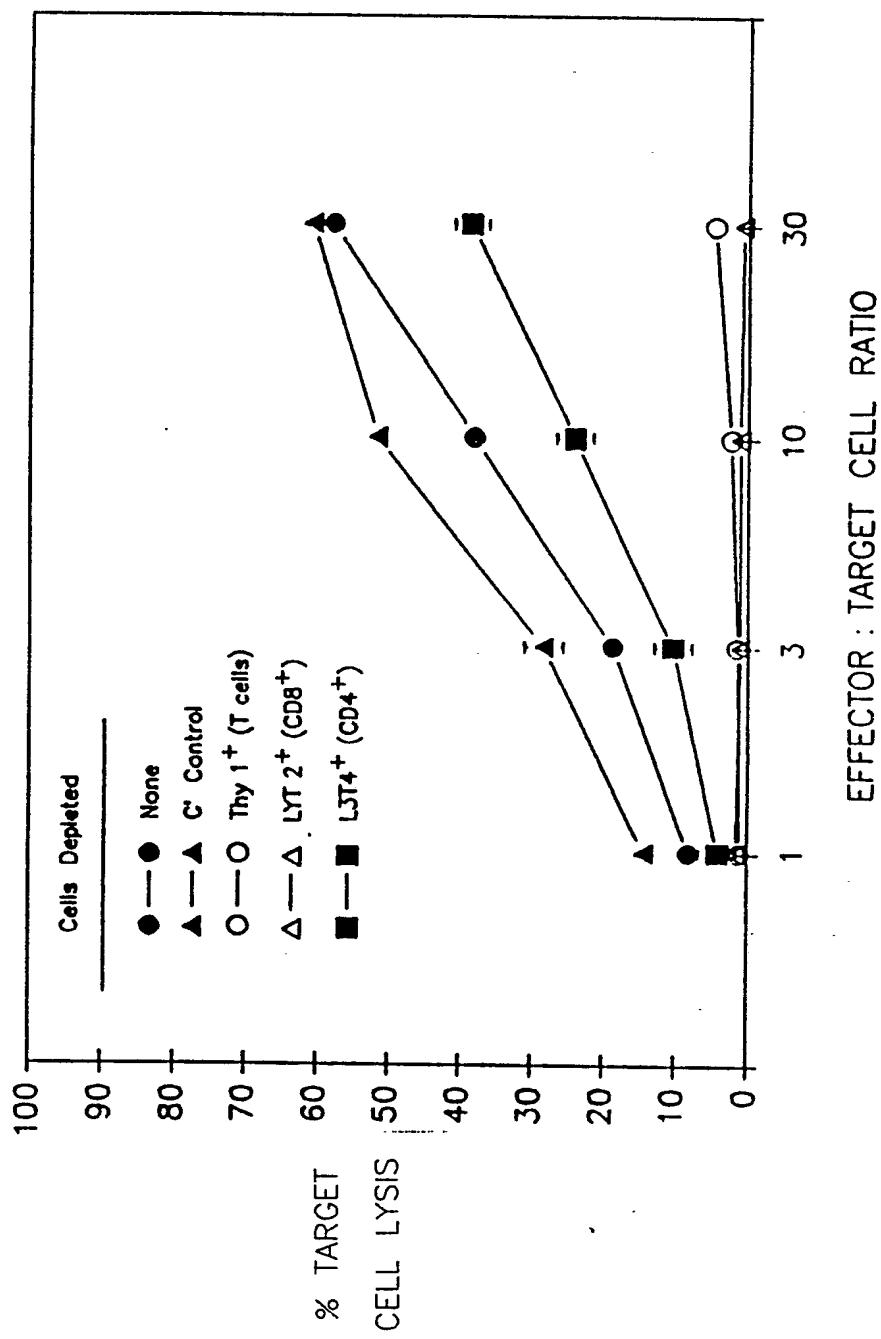
FIG. 4D

MHC RESTRICTION REQUIREMENTS FOR ANTI-BCenv CTL RESPONSE :
INHIBITION BY POLYCLONAL ANTI-MHC ANTISERA

E:T = 1:1 (42%)
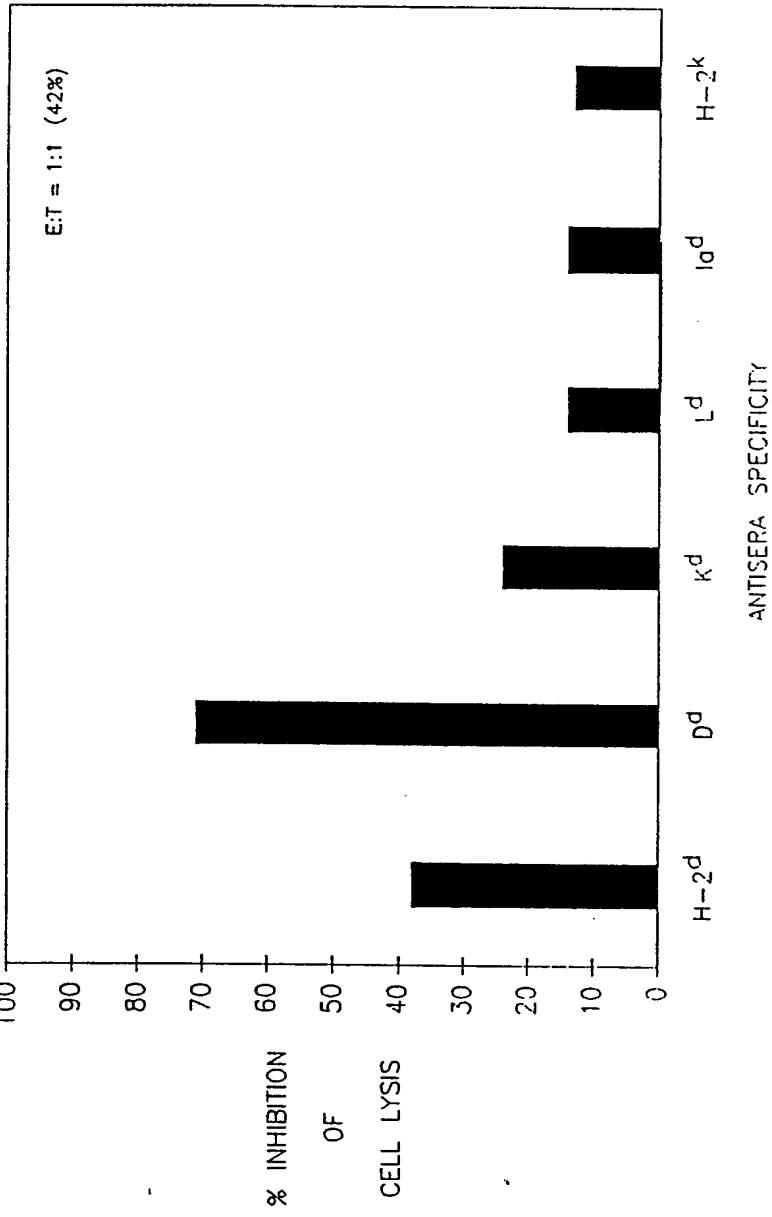
FIG. 4E

ANTI-BCenv CTL INDUCTION :

IRRADIATED VS. NON-IRRADIATED STIMULATOR CELLS
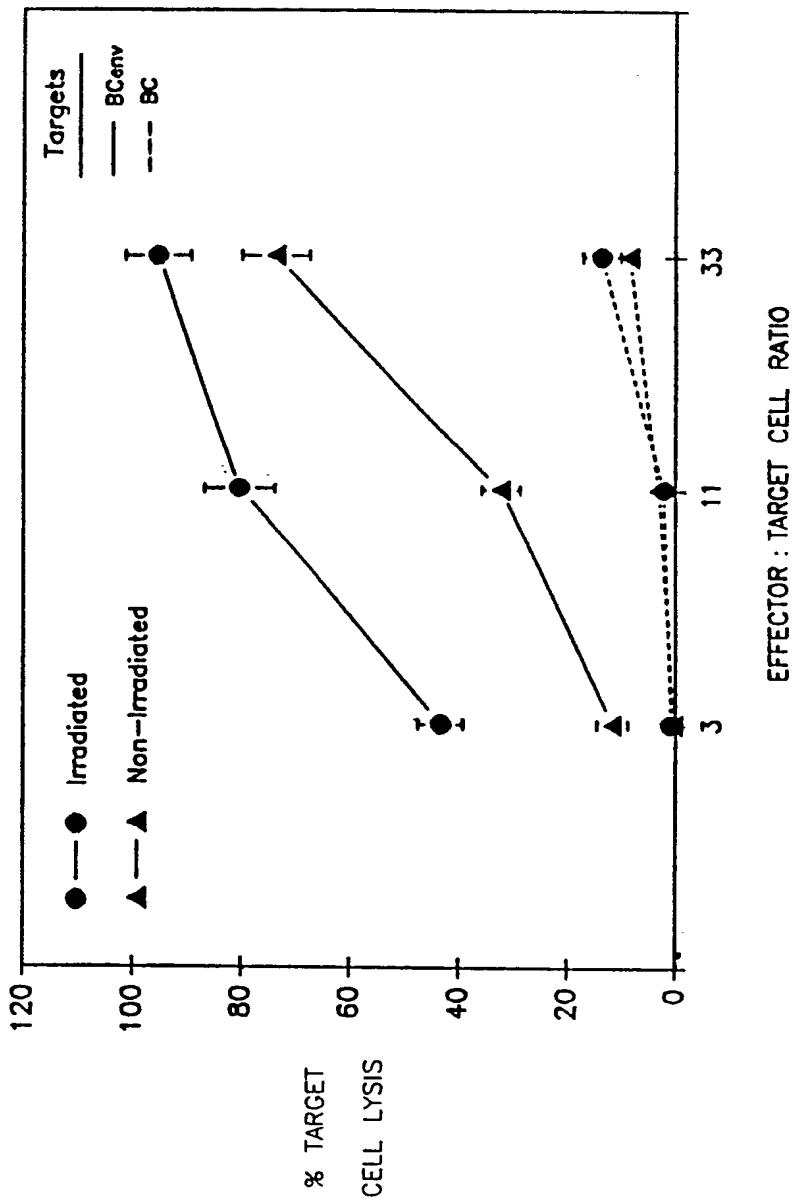
CTL INDUCTION IN BALB/C MICE IMMUNIZED WITH
VARYING CONCENTRATIONS OF BCenv STIMULATOR CELLS
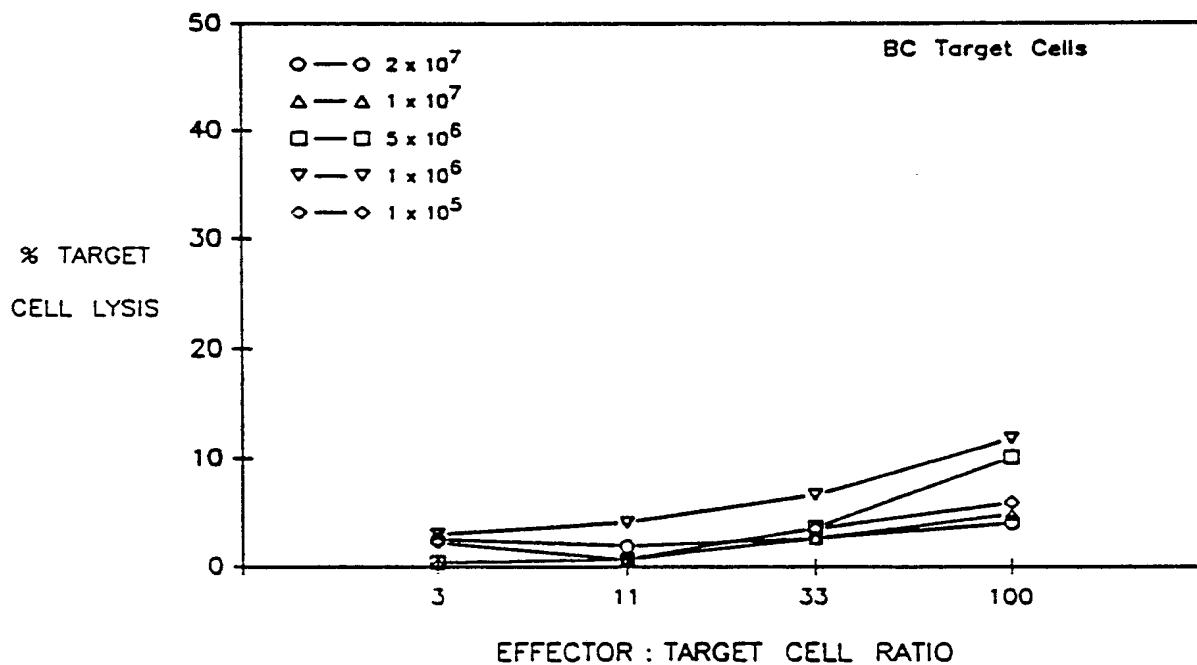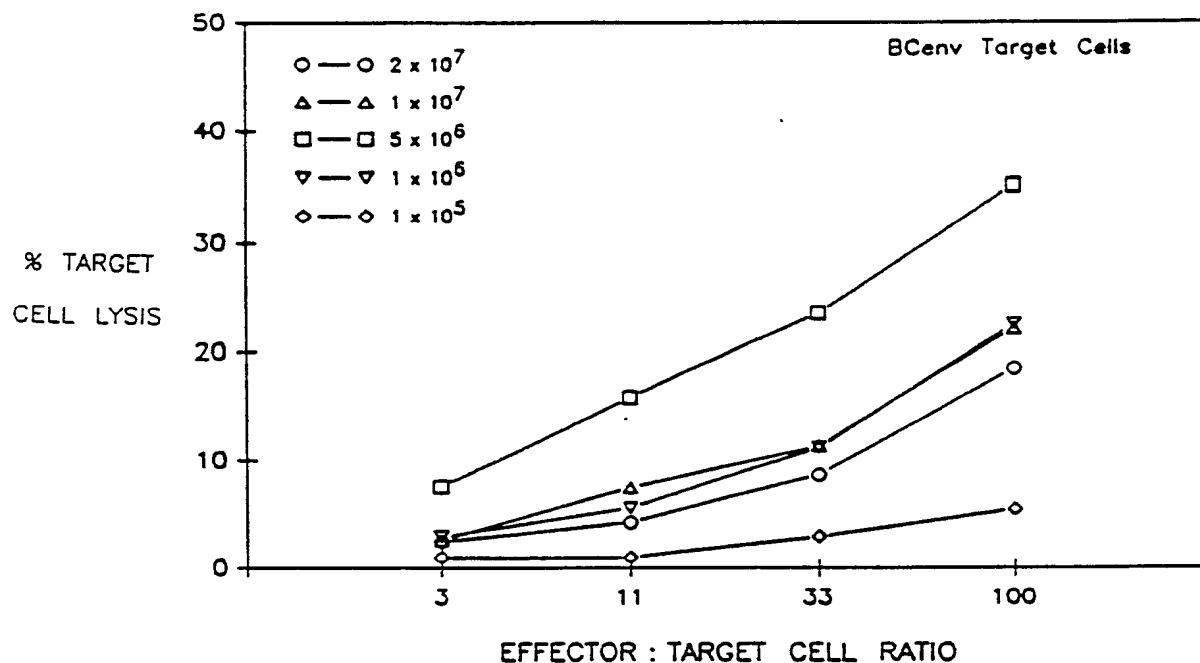
FIG. 4F

CTL INDUCTION IN DIFFERENT H-2^d MOUSE STRAINS
IMMUNIZED WITH 1 x 10^7 BC10MEenv-29 CELLS
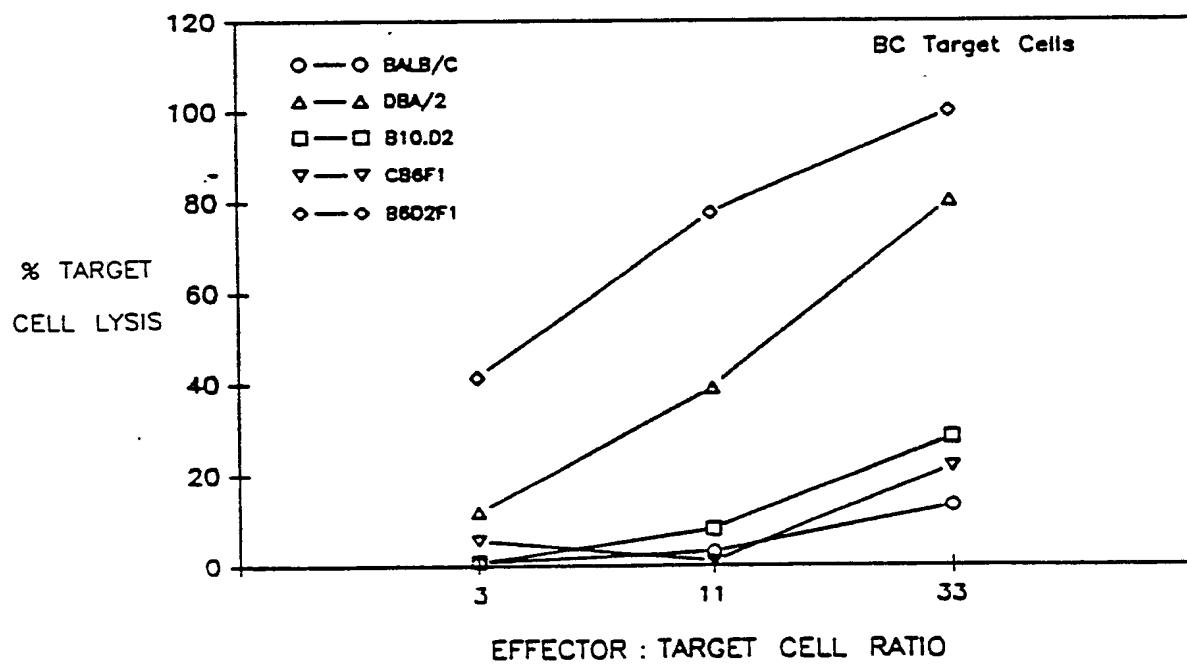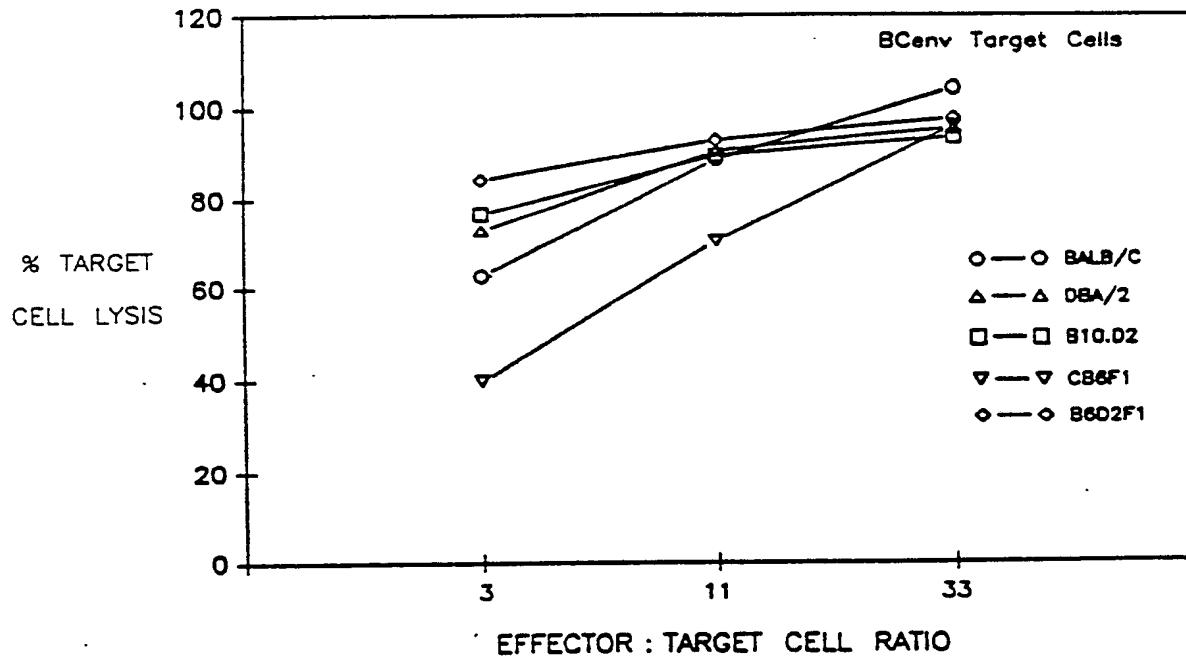
FIG. 4G

CROSSREACTIVITY OF BCenv-INDUCED CTL

FIG. 4H

Induction of Env Specific CTLs With
Transiently Transfected BC pCMVenv IIIb

FIG. 4I

-13/39-
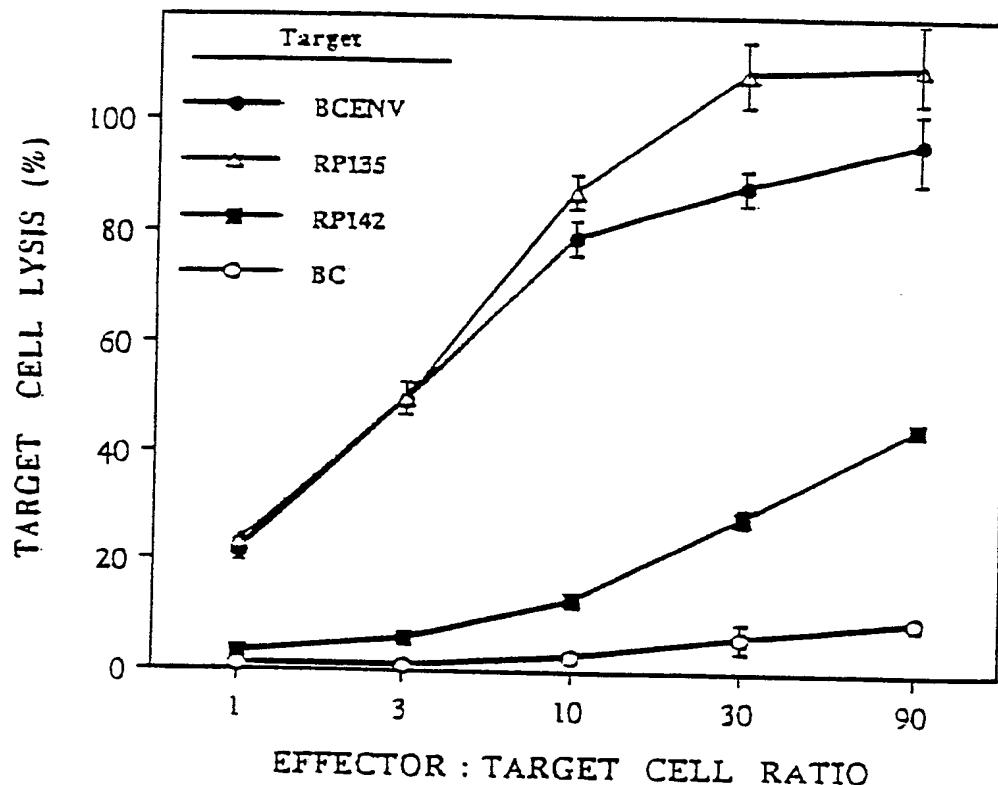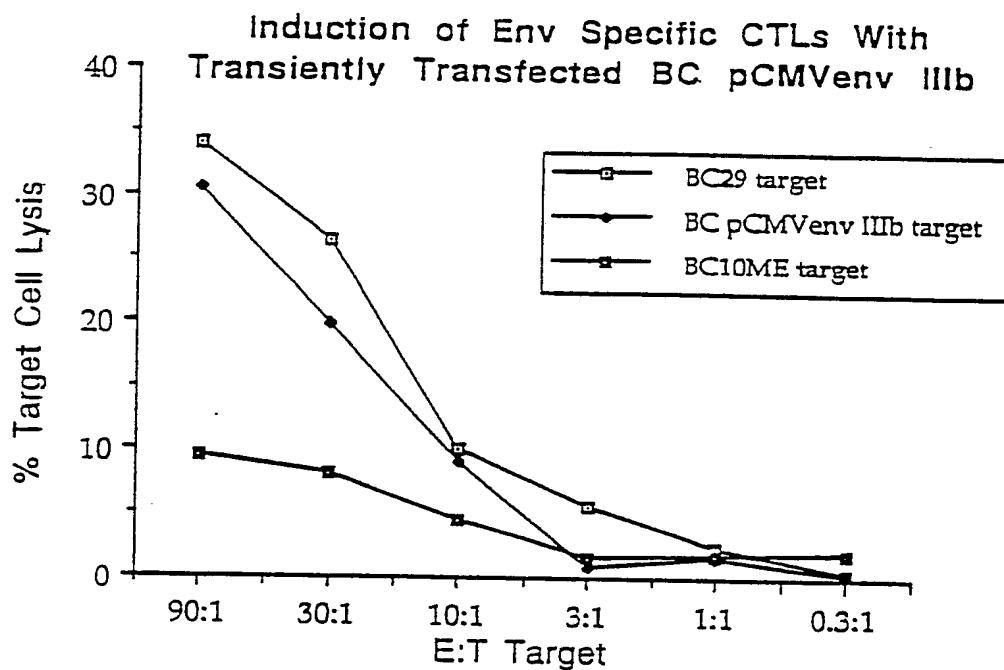
Transient Infection for Constructing Target Cells

C57BL/6 anti-I12-Dd CTL on MIIC-Dd transfected murine BC target cells

FIG. 4J
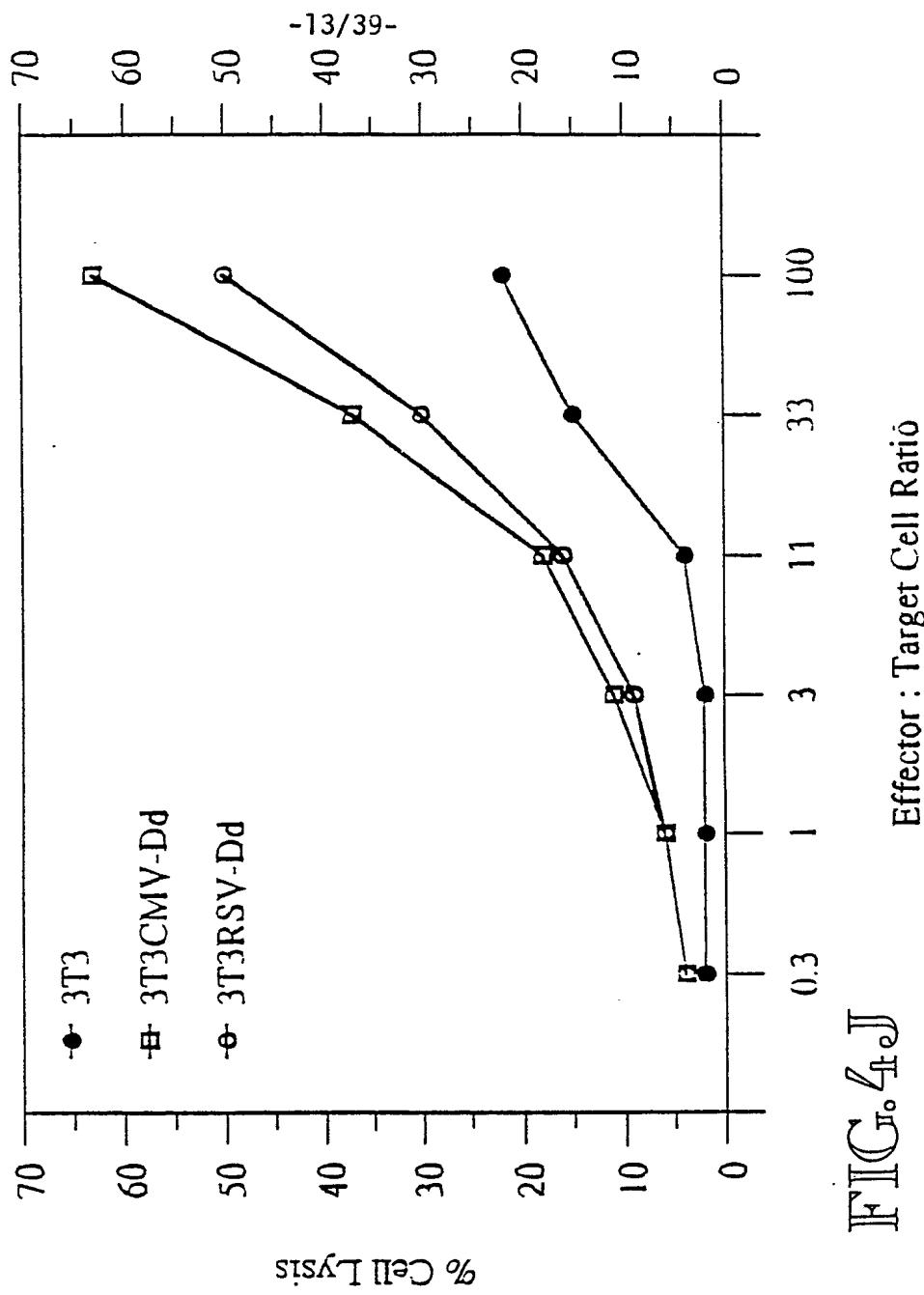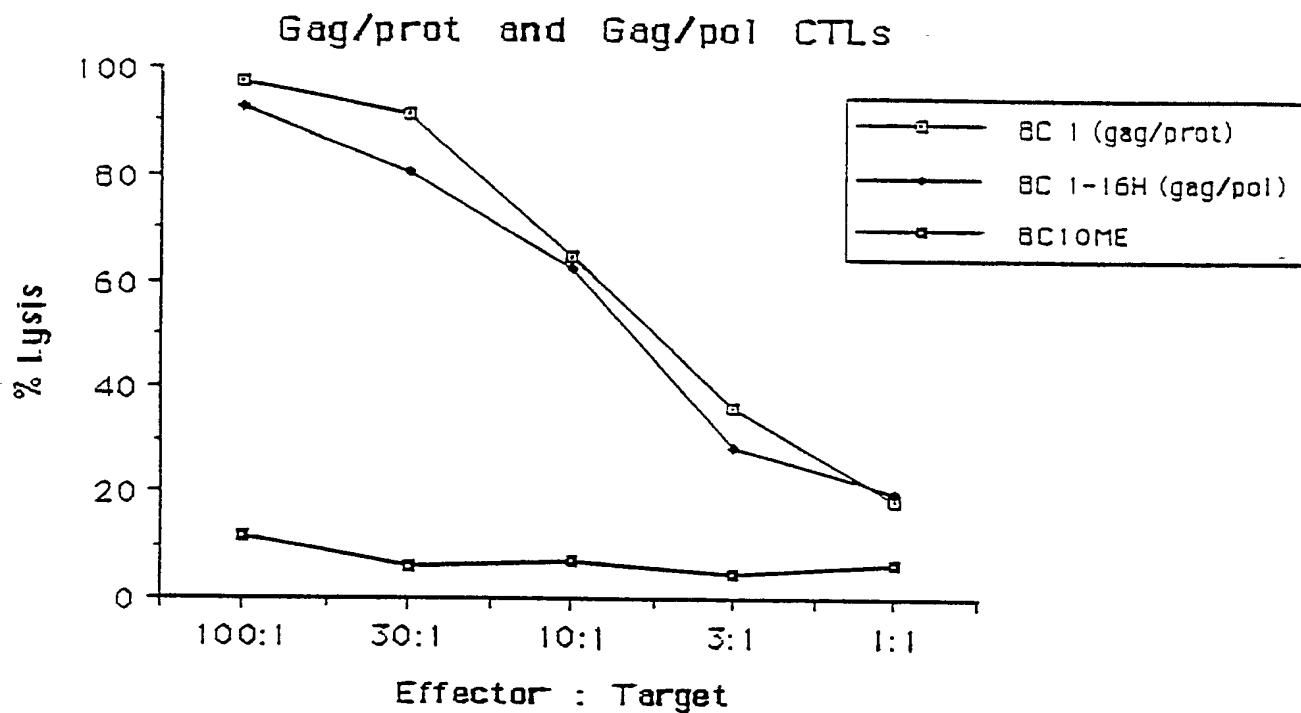
FIG. 4K
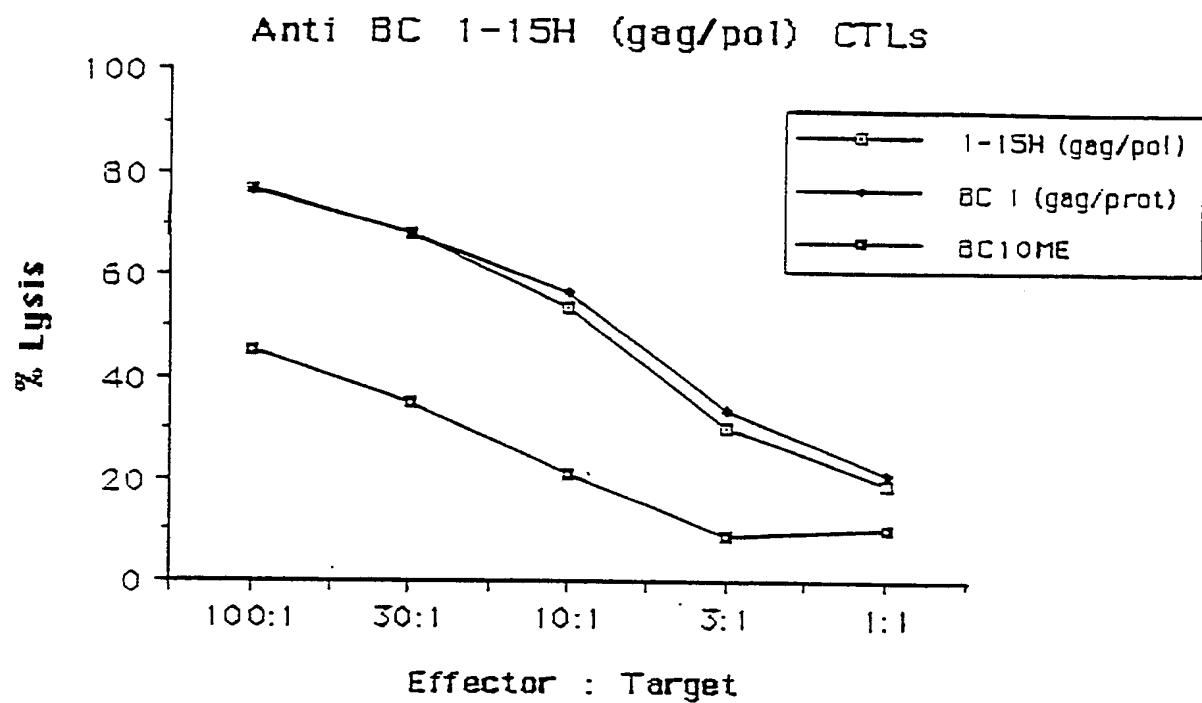
FIG. 4L
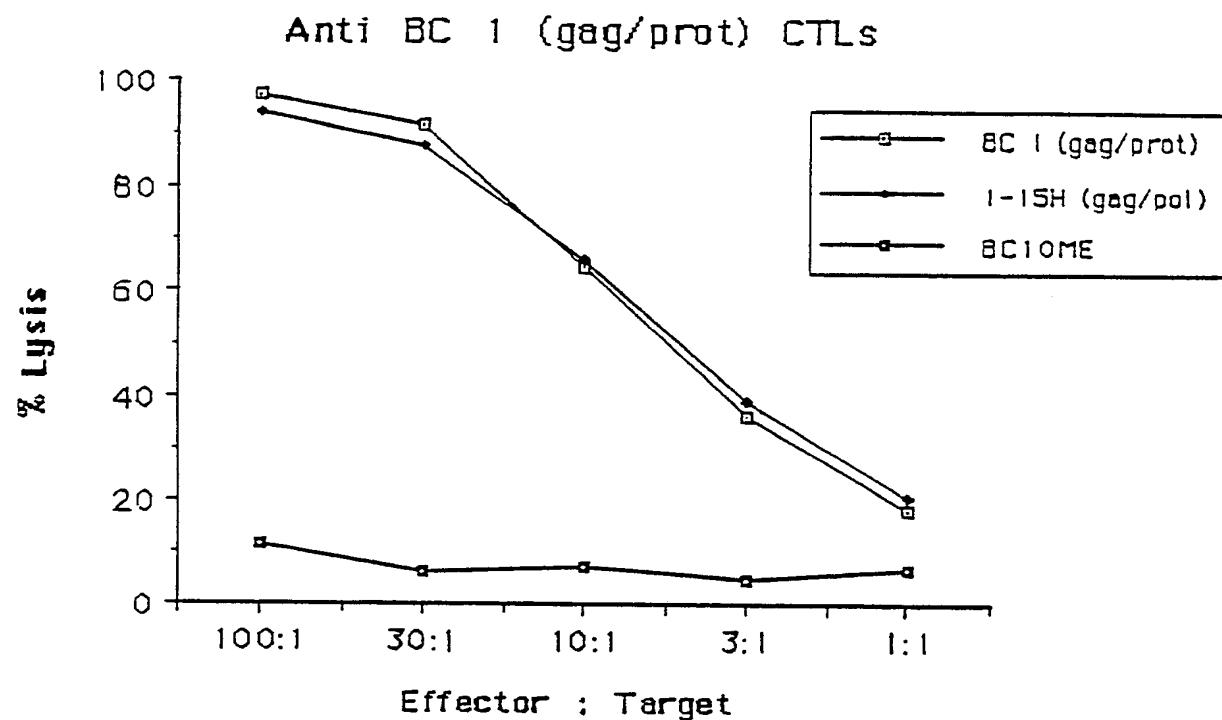
FIG. 4M
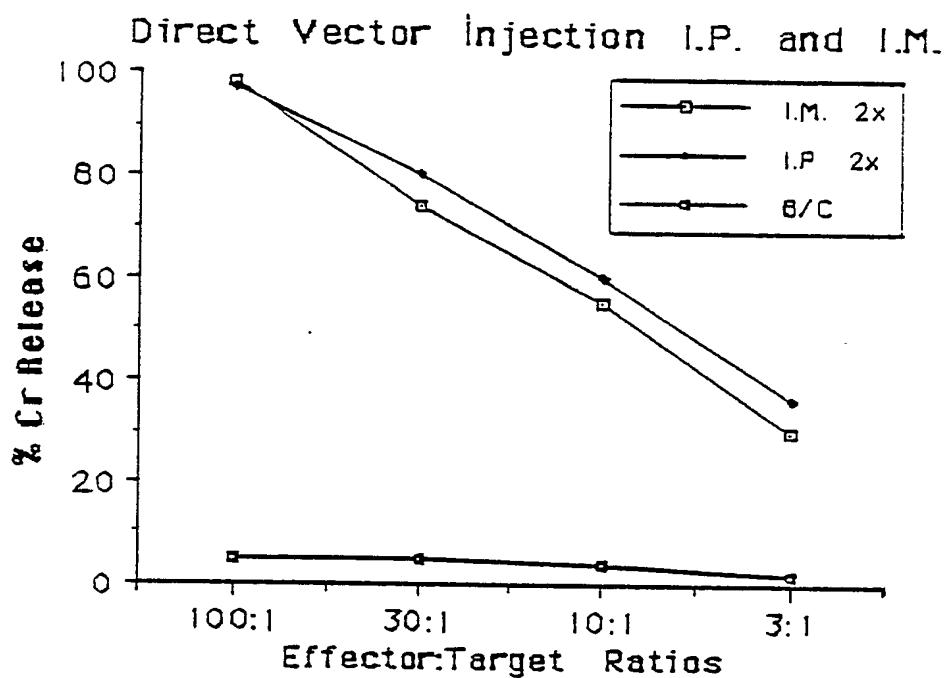
FIG. 4N
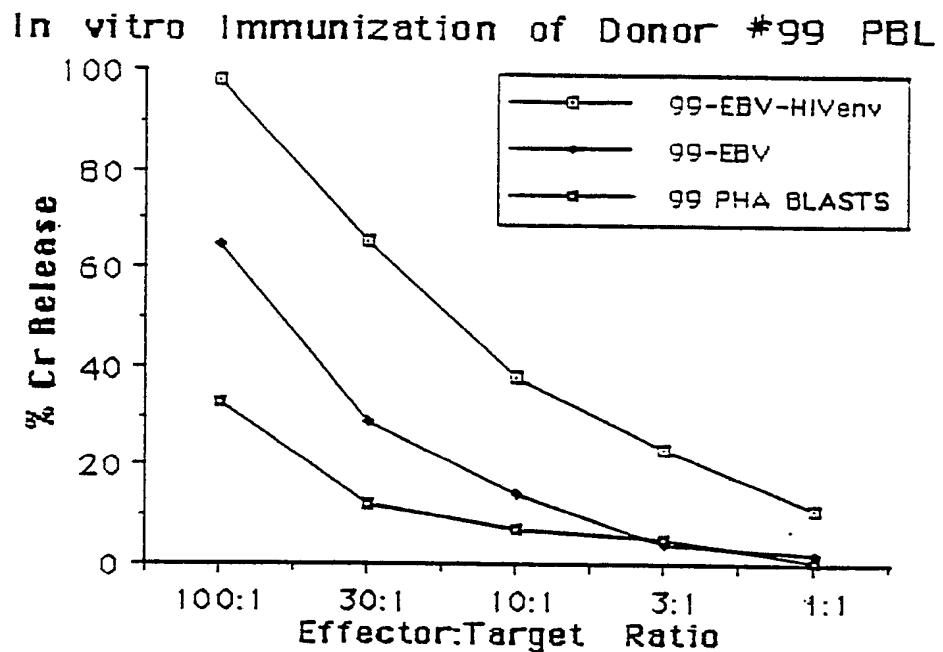
FIG. 40

INFECTION OF TARGET CELLS WITH MHC AND ANTIGEN.
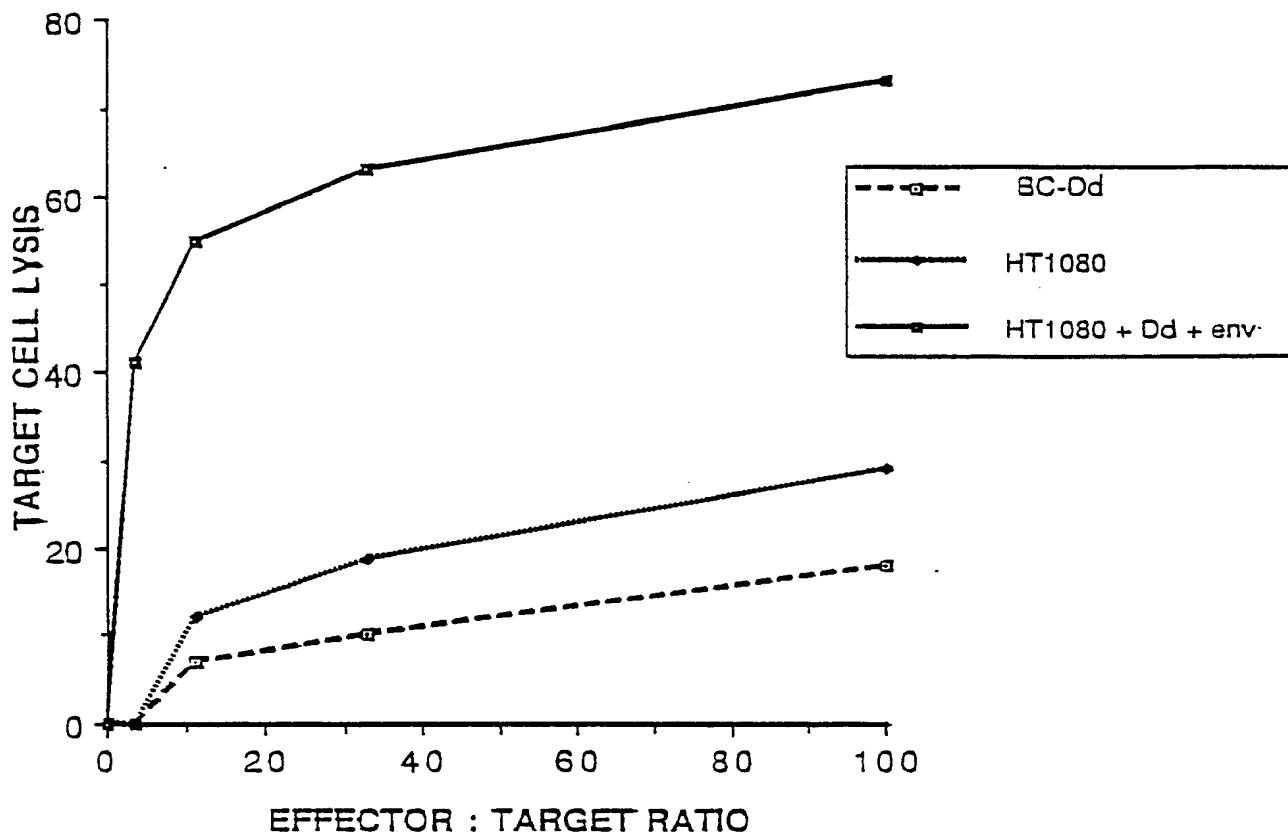
FIG. 4P

A.   CTL Induction with BCenv
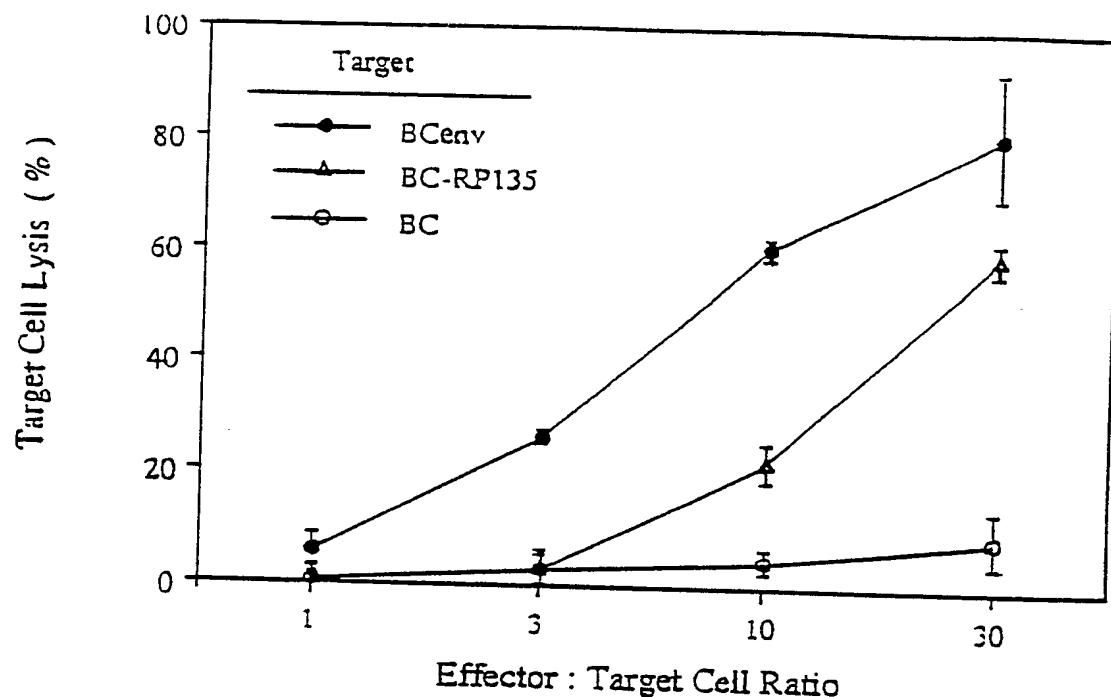
B.   CTL Induction with BCenvΔV3
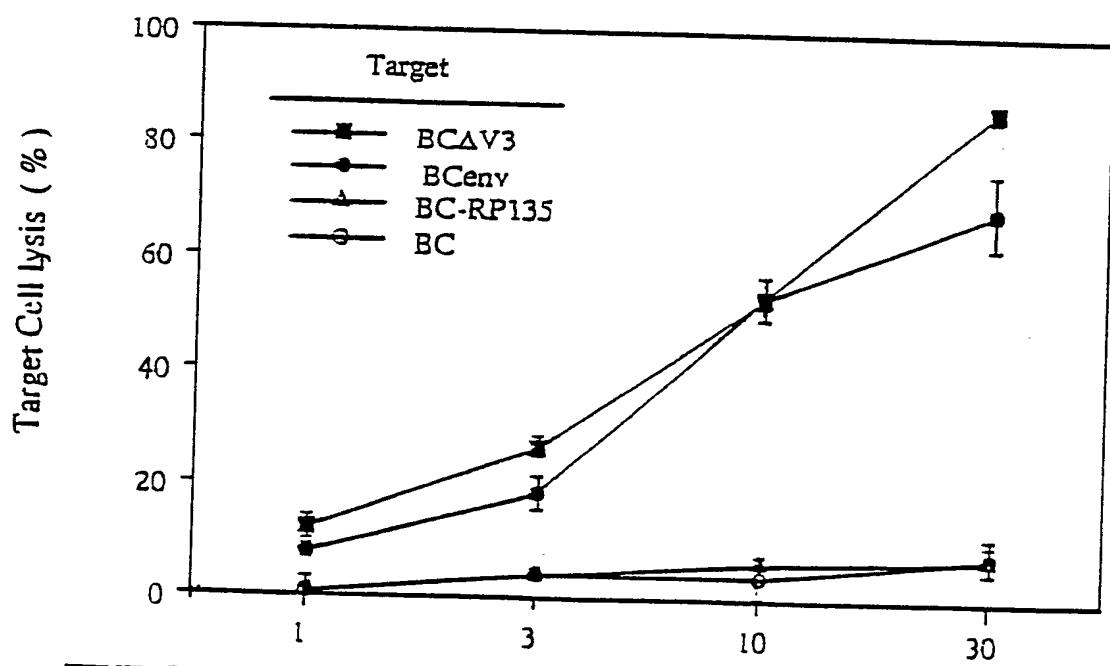
FIG. 4Q

A. Lysis of BCpCMV Chunk 1 Target
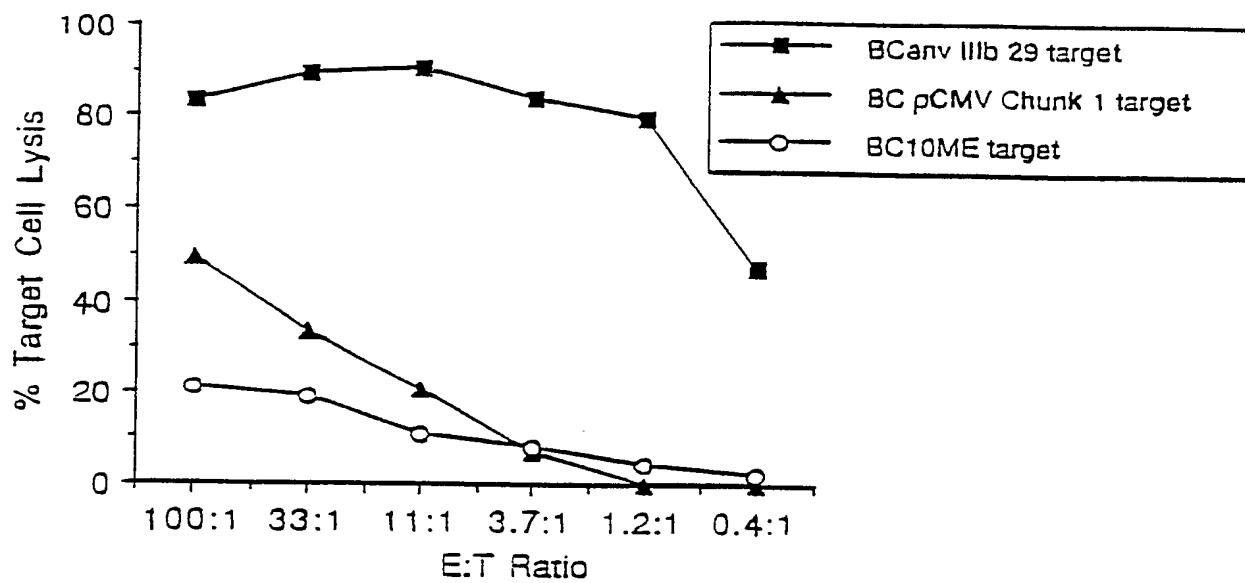
B. Induction of Balb/c anti-Chunk 1 CTLs
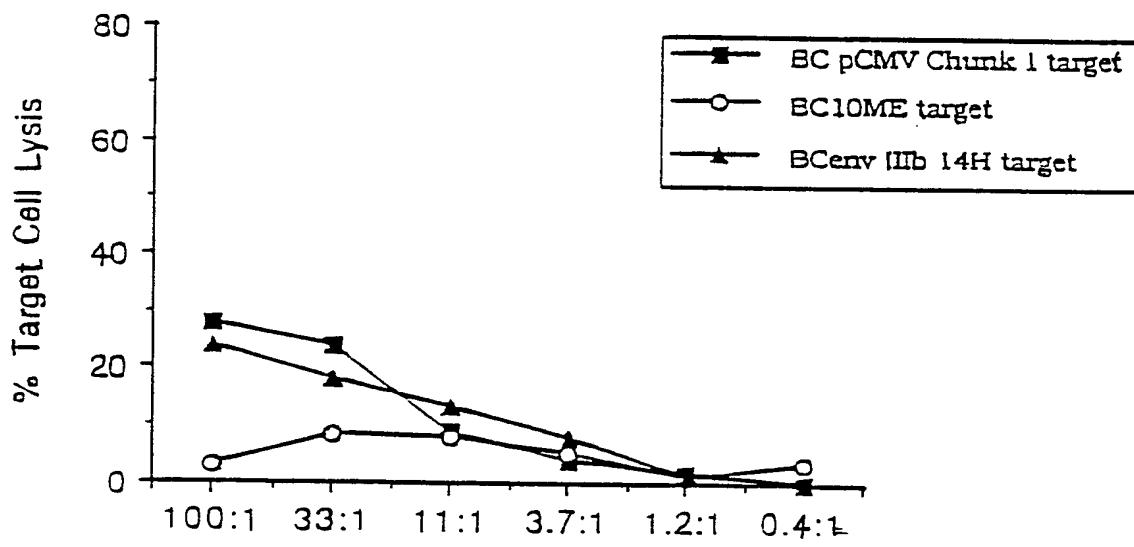
FIG. 4R

RECEPTOR BLOCKER VECTOR
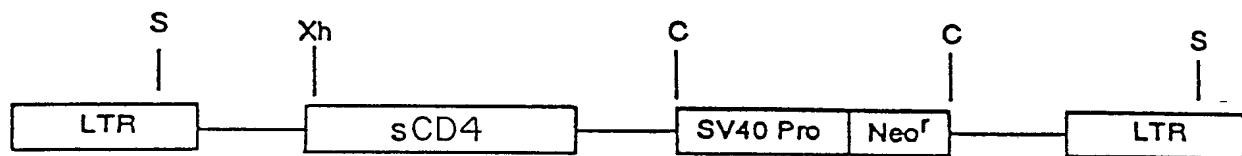
FIG.5

Construction of retroviral vectors pTK - 1 and pTK - 3
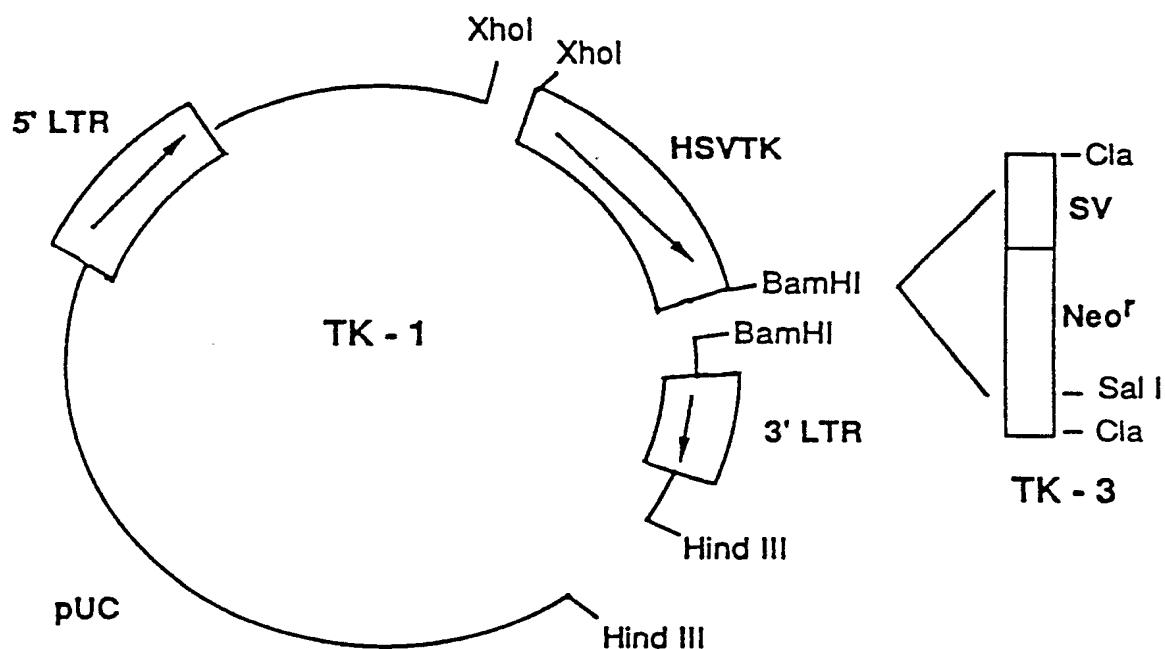
FIG. 6

Construction of HIV-conditionally - lethal vector KTVIHAX
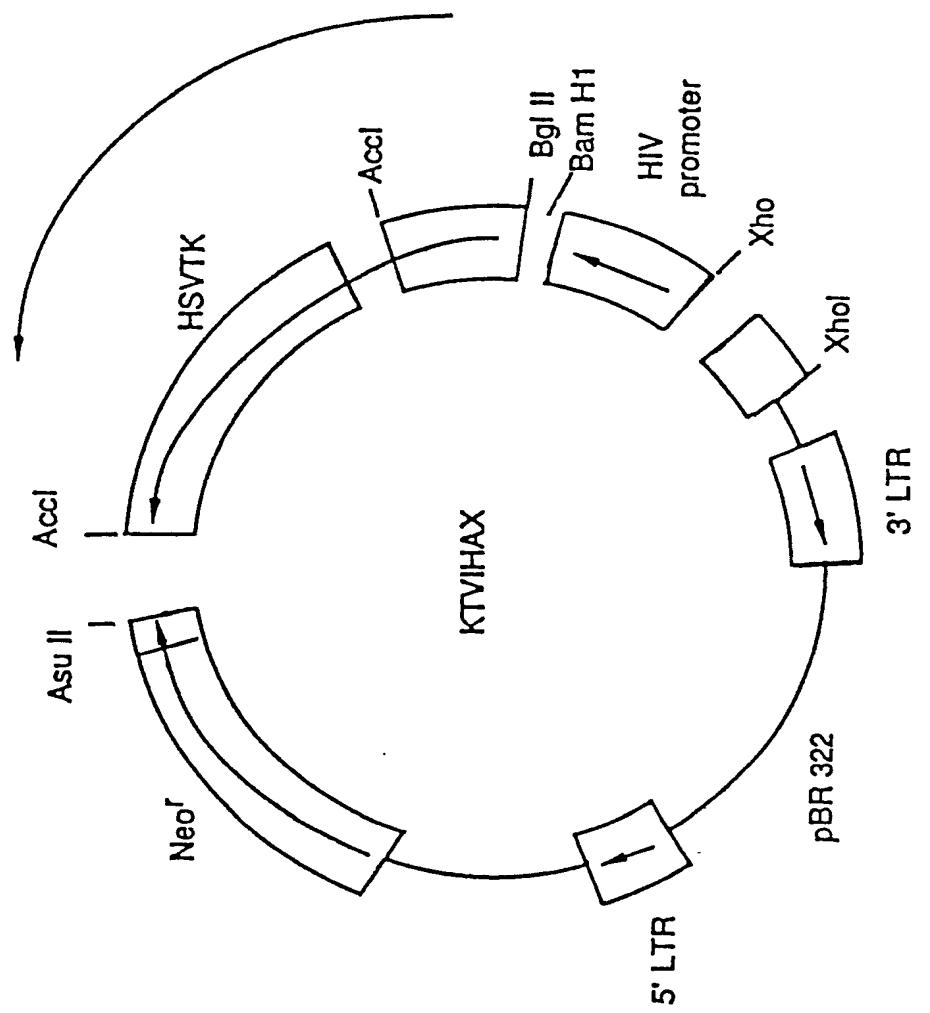
FIG. 7

Construction of KTVIH5 and KTVIH Neo
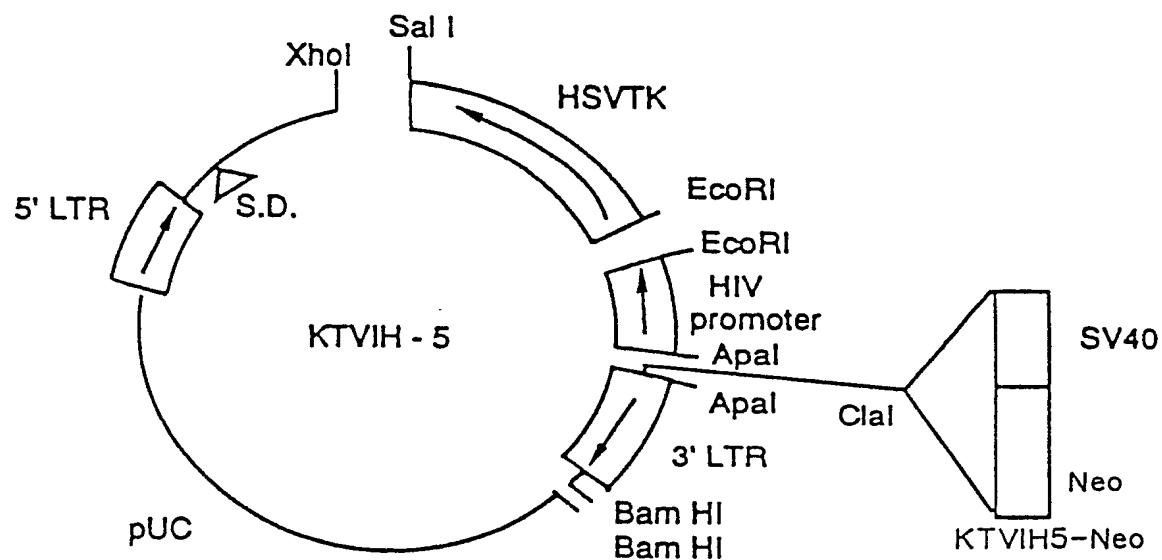
FIG. 8

CONSTRUCTION OF MHMTKNEO RETROVIRAL VECTOR
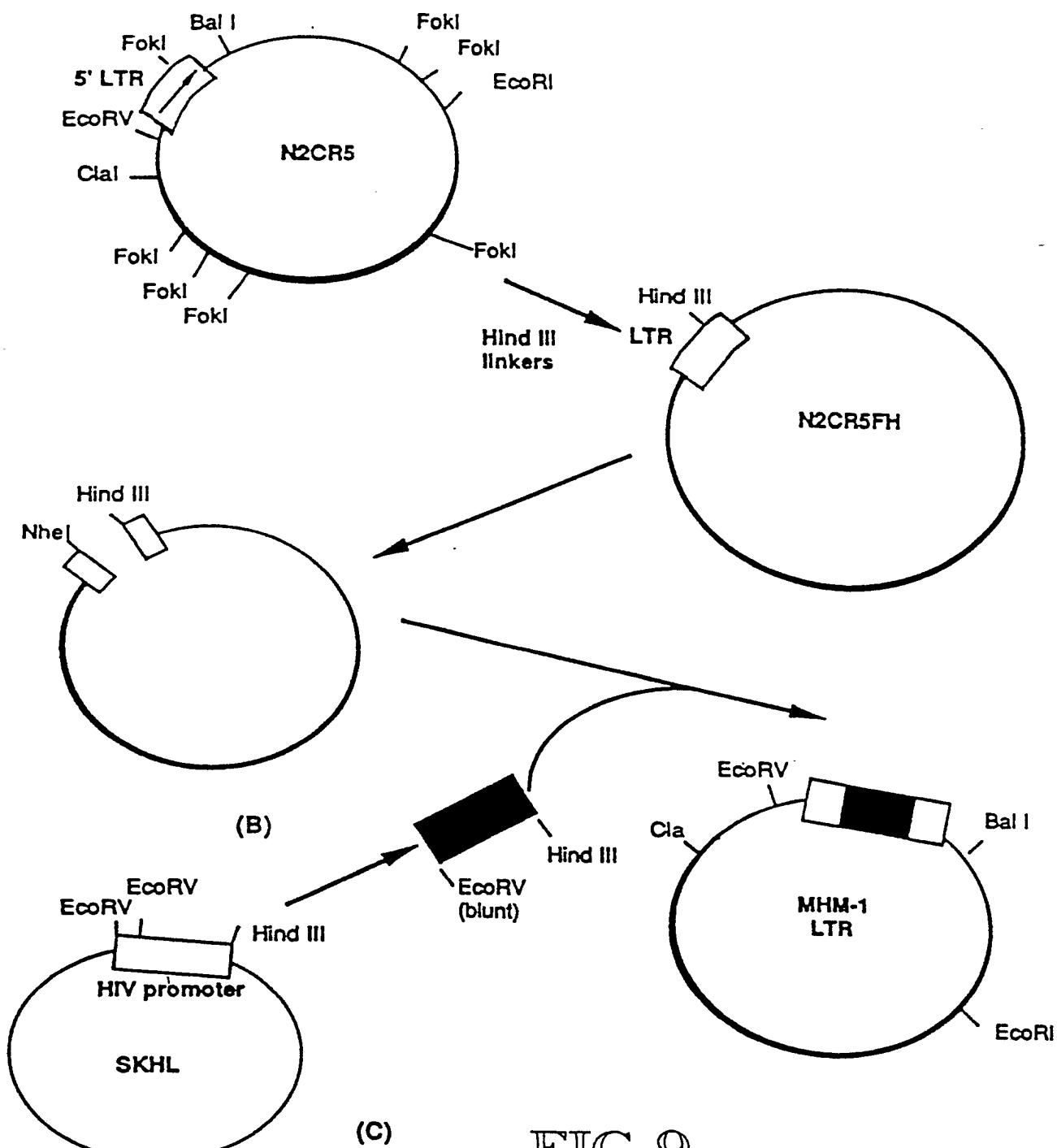
FIG. 9

FIG. 9 CONT.
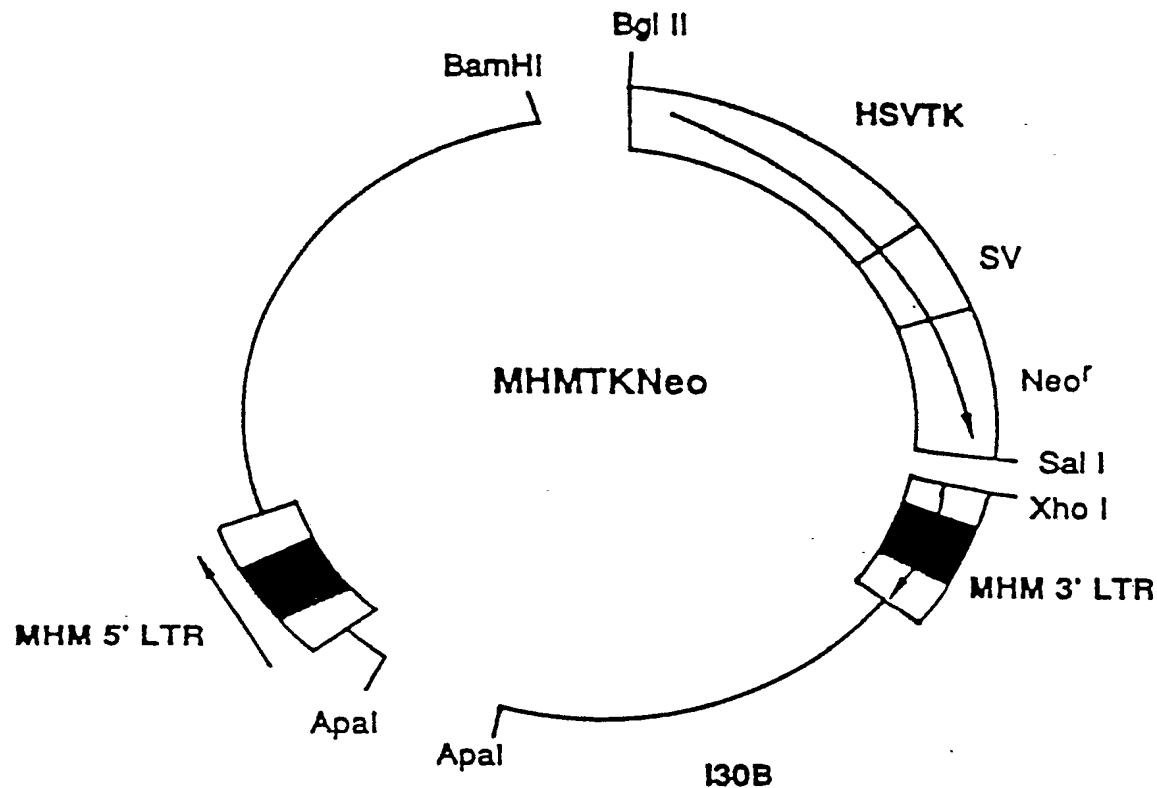
Construction of HIV tat- and HIV rev-responsive conditionally lethal vector, RRKTVIH

FIG. 10

FIG. 11

FIG. 12
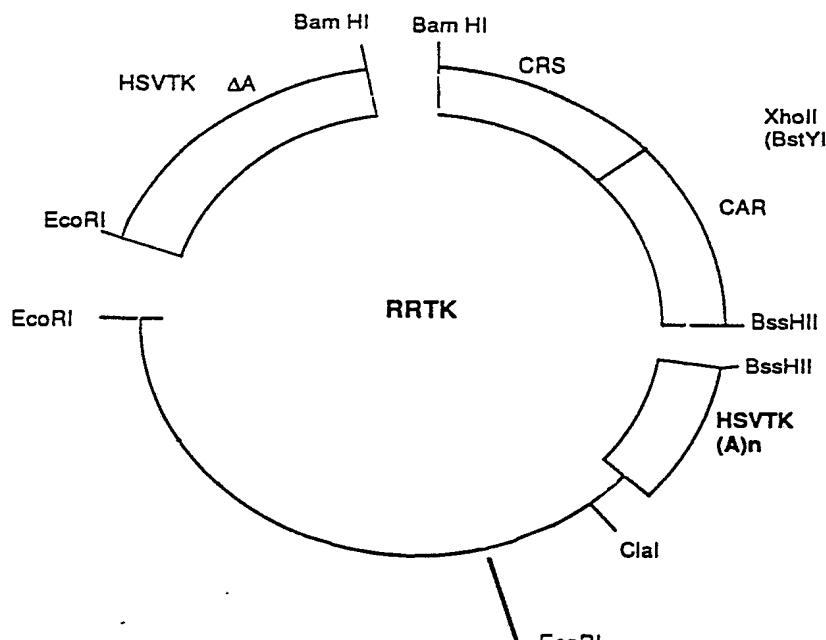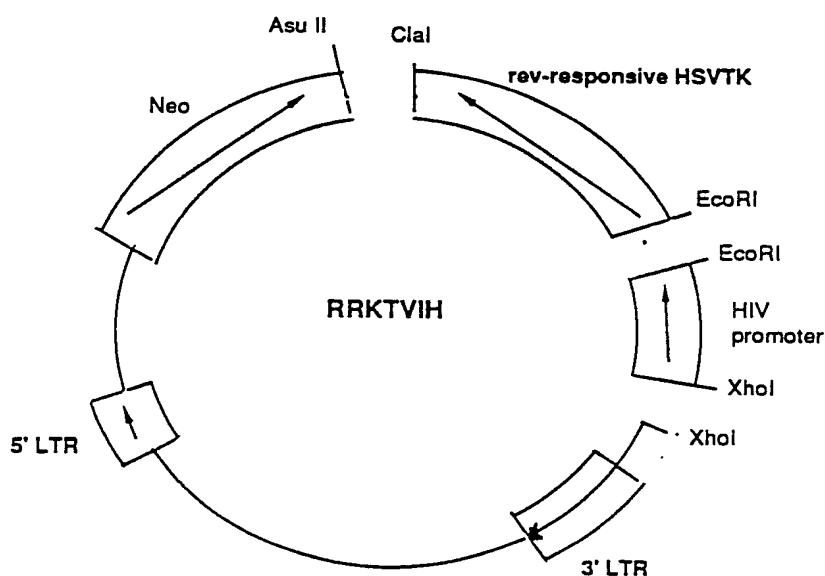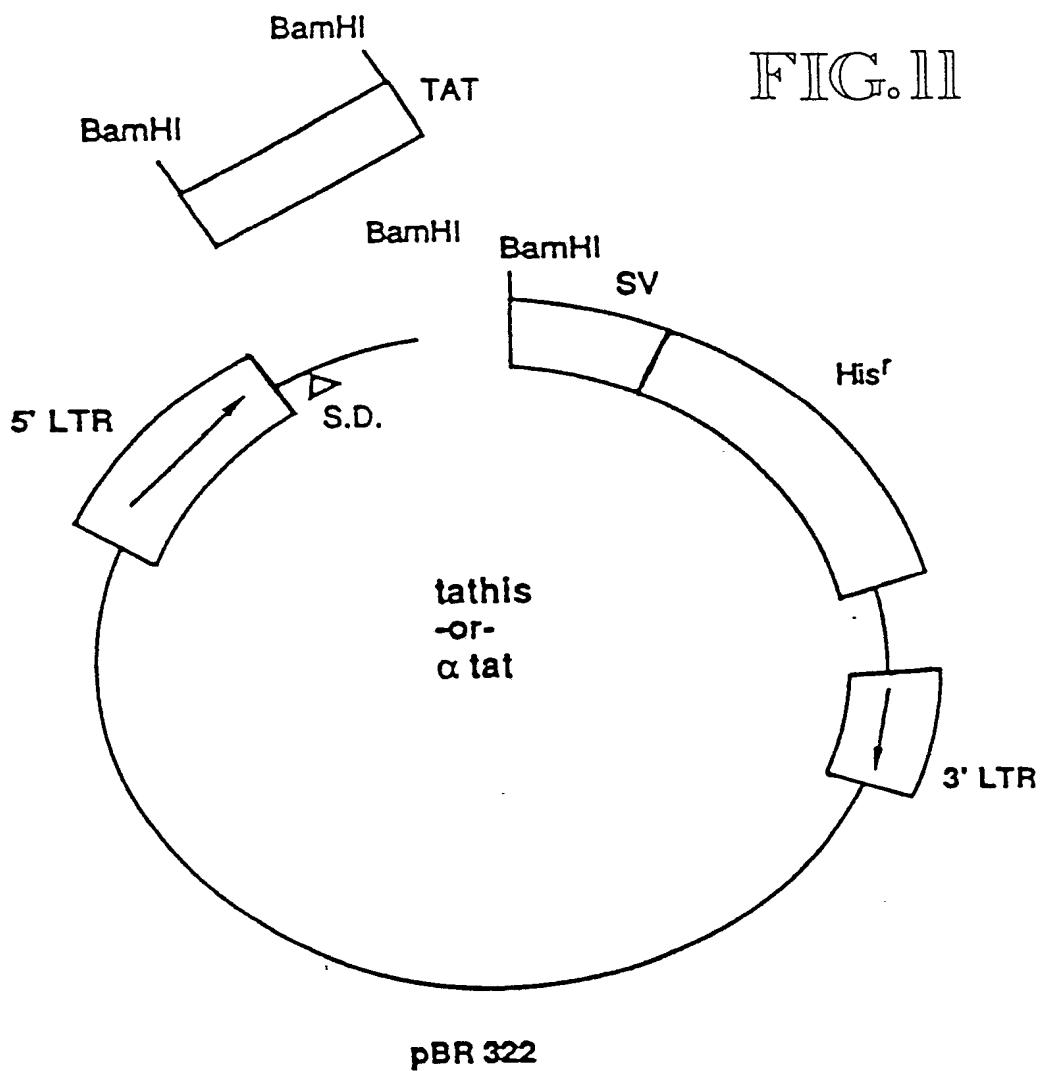
ACV Toxicity in Cells Containing Conditional Lethal Vectors

FIG. 13

FIG. 14

1. Mo. LTR — 0.7 kb
2. SV2neo — 1.8 kb
3. HIV LTR — 0.7 kb
4. Reporter Gene
5. CRS-CAR Sequences — 2.1 kb
1. Mo. LTR — 0.7 kb

FIG. 15

6. SV40 t Intron — 0.7
2. Sv2neo — 1.8 kb
5. CRS-CAR Sequences — 2.1 kb
4. REPORTER GENE
3. HIV LTR — 0.7 kb
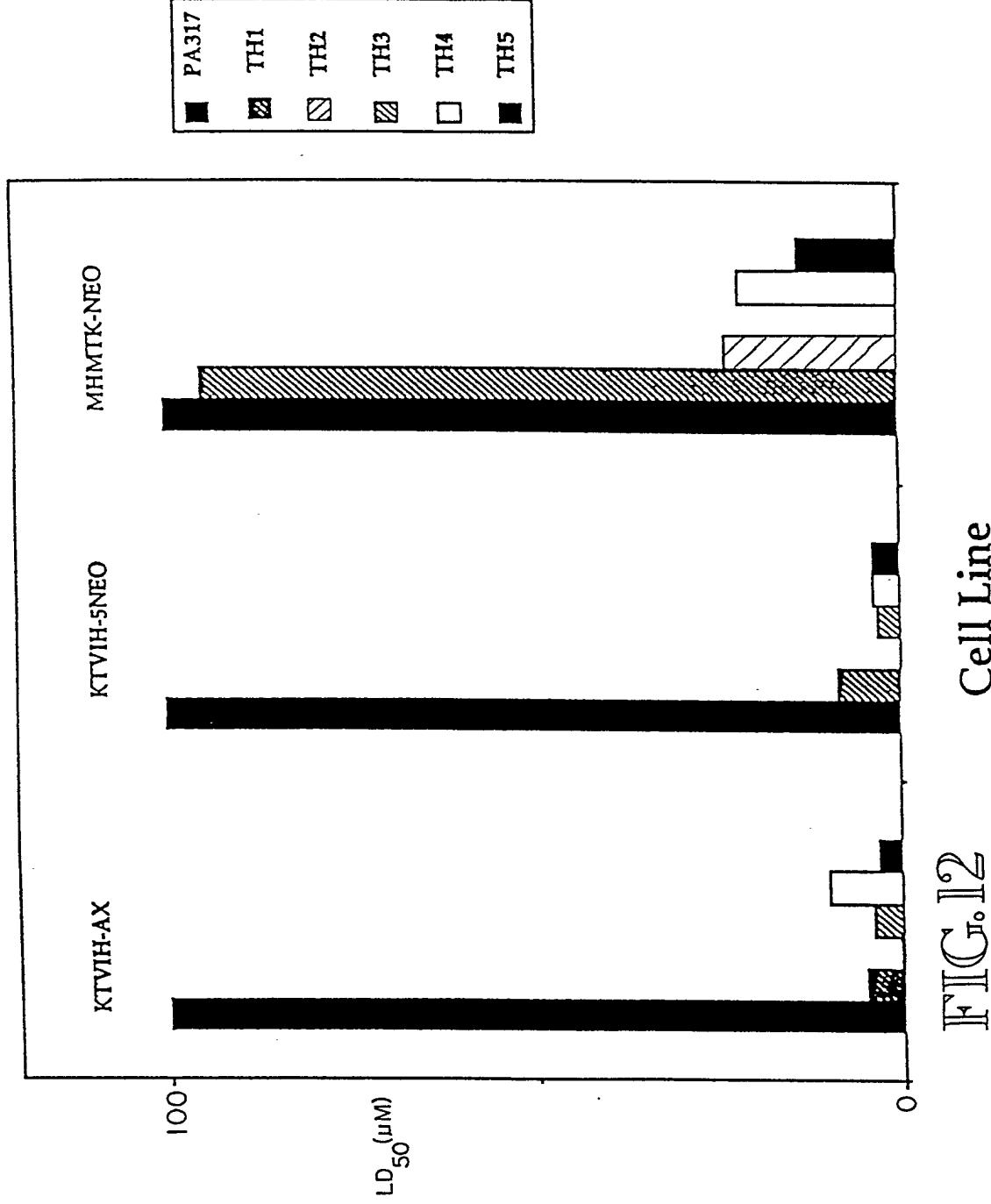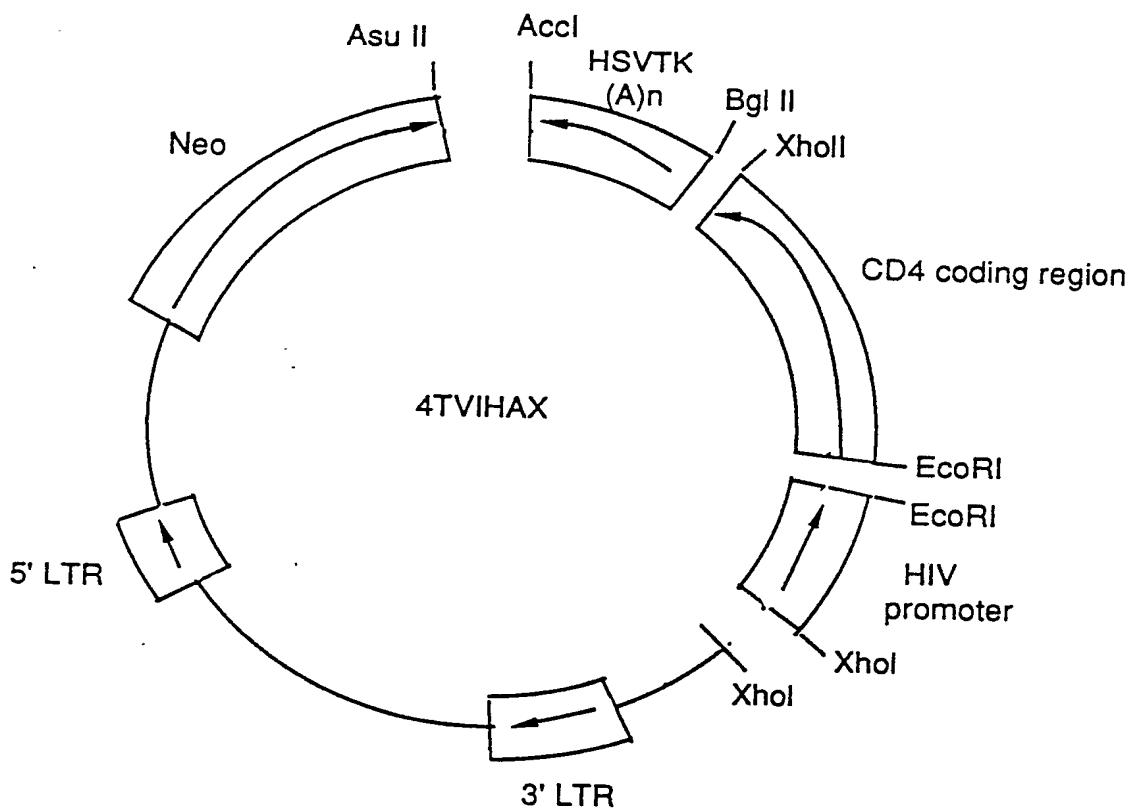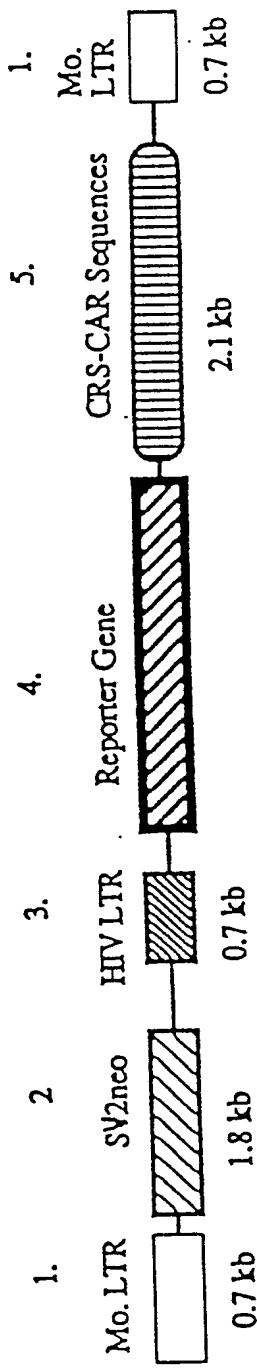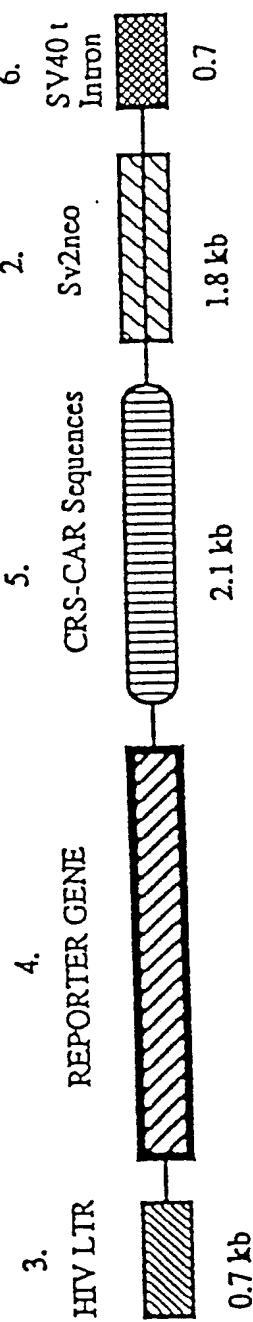
FIG. 16

EFFECT OF AZT ON HIV INFECTION
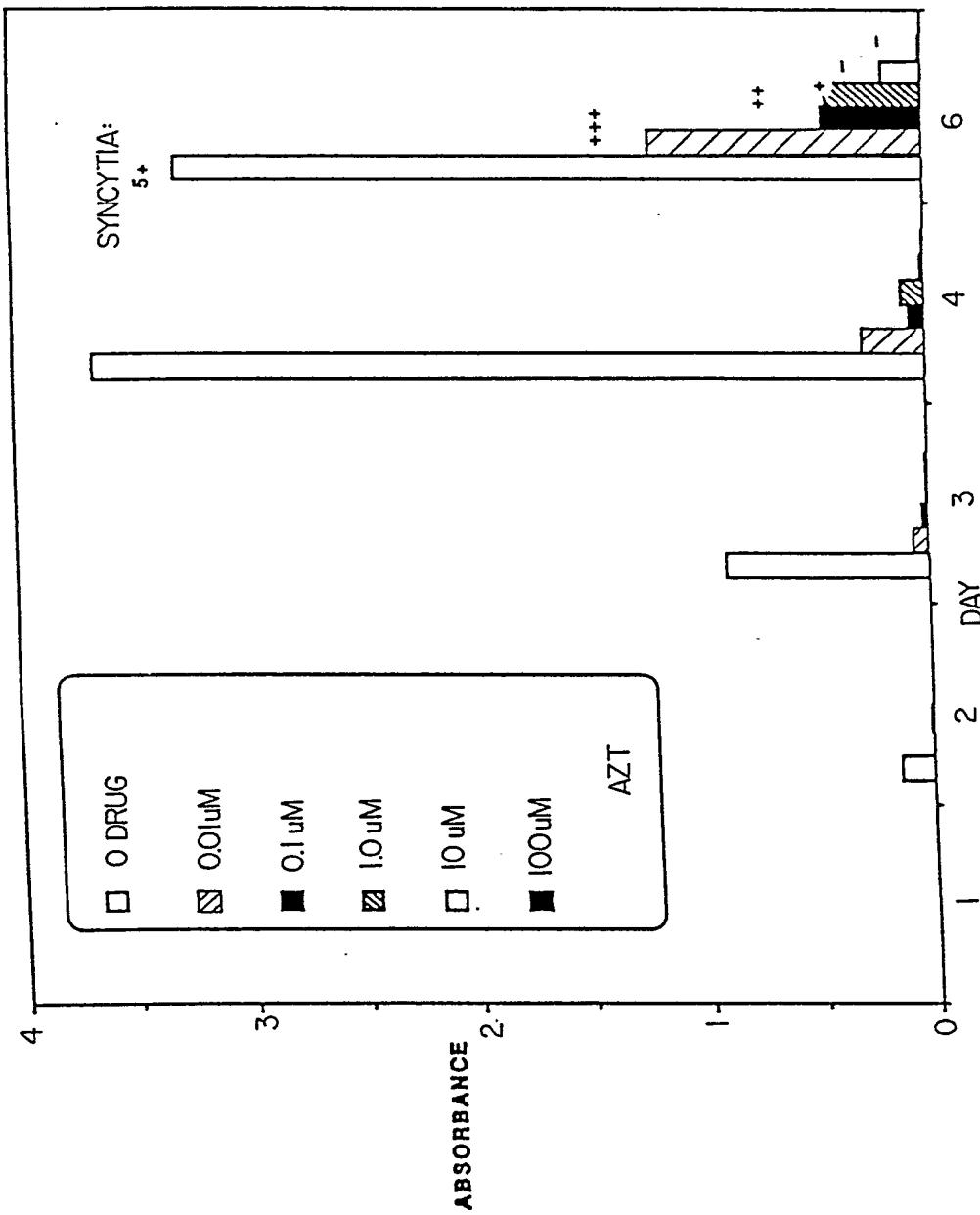
FIG. 17

EFFECT OF ddC ON HIV INFECTION

FIG. 18
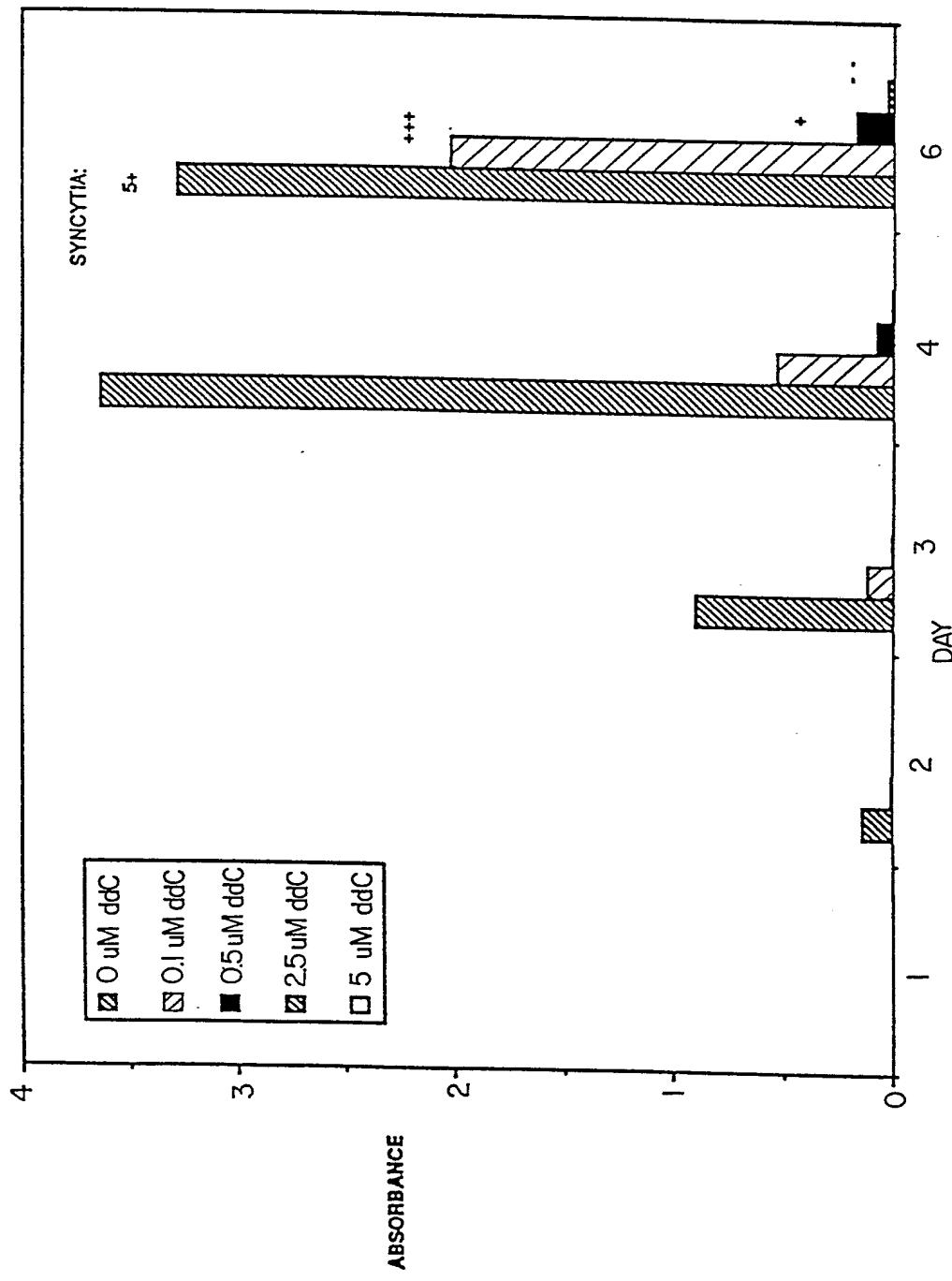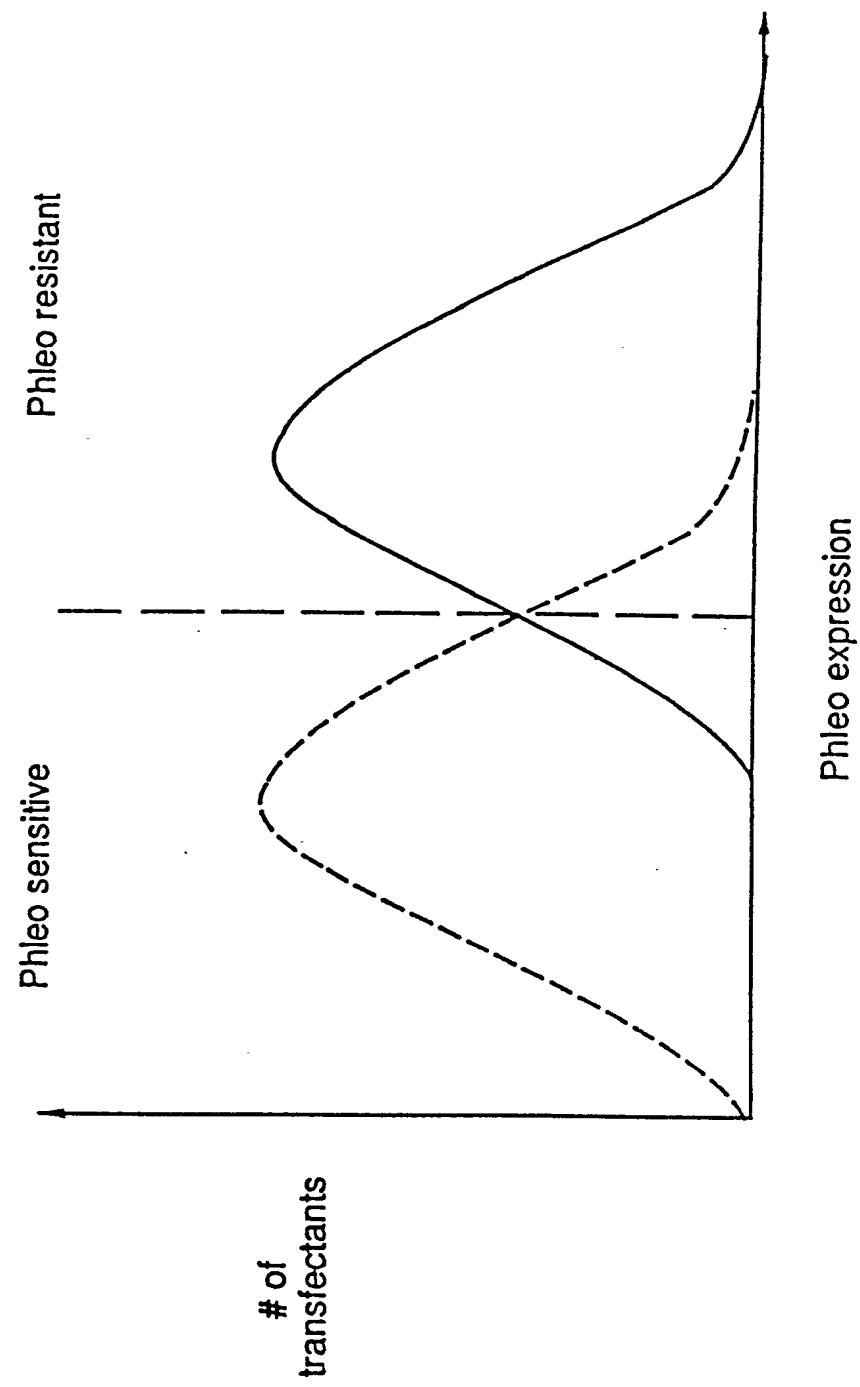
# Plasmids Designed to Increase Viral Protein Production
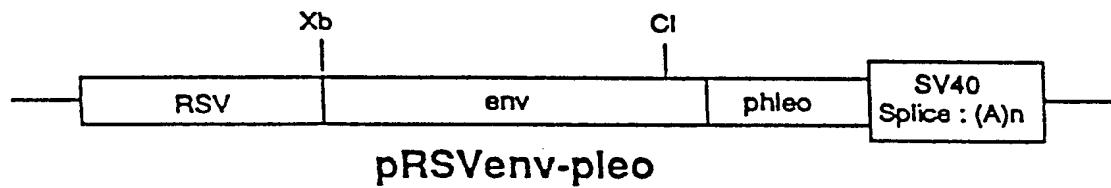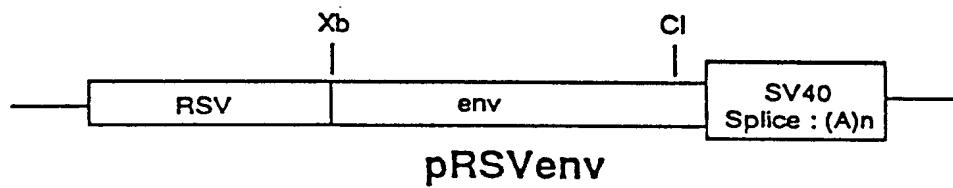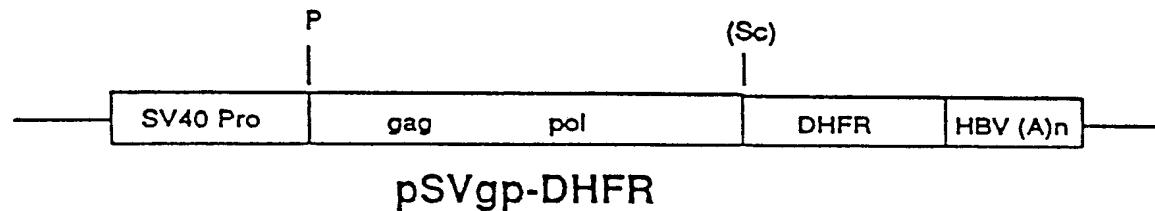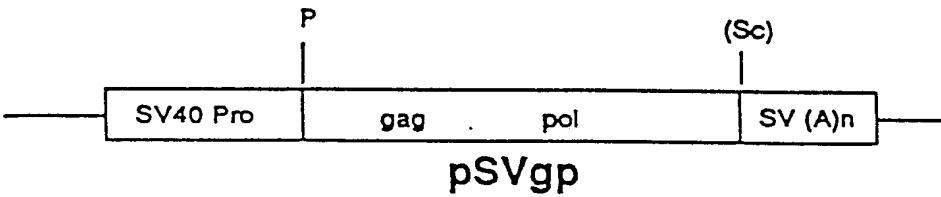
FIG. 19

CREATION OF FUSION SITES ON HIV AND MLV ENV GENES -37/39-

HIV

gp120   gp41

ST=5411   TT=8001   A=7775
SR=5449   TR=8230   B=7858
                                              C=7946

MoMLV

gp70   p15E
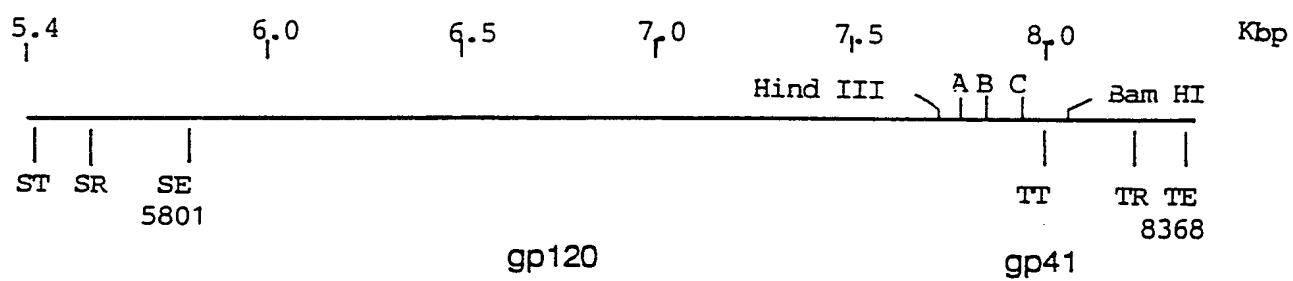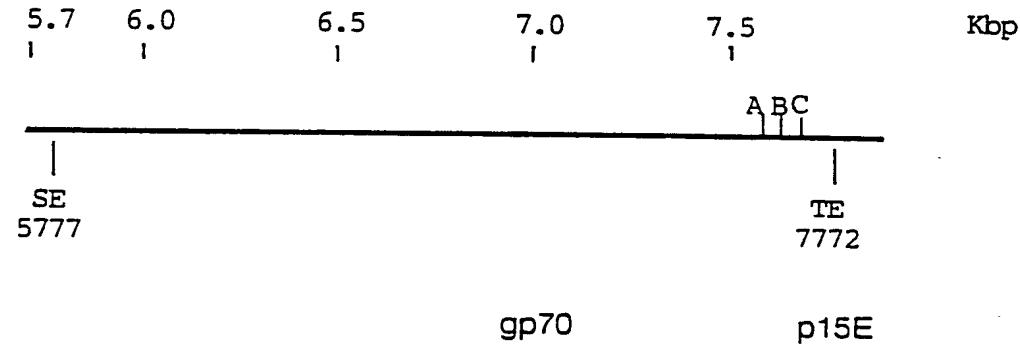
A=7585
B=7630
C=7675

FIG. 20

A. Normal 5' LTR

| U3 | R | U5 |
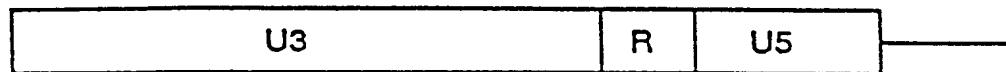
B. Hybrid 5' LTR

Bssh II (-52) or
Sacl (-35)

| Heterologous Promoter | | R | U5 |

FIG. 21

FIG. 22